

**NUCLEIC ACID ENCODING AN AVIAN *E. COLI* ISS  
POLYPEPTIDE AND METHODS OF USE**

**Continuing Application Data**

5           This application is a continuation-in-part of U.S. Patent Application Serial  
No. 09/282,352, filed March 31, 1999, which is a divisional of U.S. Patent  
Application Serial No. 09/023,221, filed February 12, 1998, now U.S. Patent  
6,087,128, issued July 11, 2000, each of which is incorporated by reference herein.

10                           **Government Funding**

          The present invention was made with government support under Grant No.  
1P20RR11827-01, awarded by National Institutes of Health. The Government has  
certain rights in this invention.

15                           **Background of the Invention**

          Pathogenic *Escherichia coli* ("*E. coli*") is the causative agent of a variety of  
diseases in humans and animals, and often have the ability to avoid, resist or  
inactivate a multi-cellular organisms chemical and cellular defenses for a significant  
period of time after the host has been exposed to the pathogen. *E. coli* infection of  
20   the avian respiratory tract causes respiratory tract lesions and septicemia.

*E. coli* infections are often secondary to infections of birds by infectious  
bronchitis virus, Newcastle disease virus and *Mycoplasma* spp (Gross, Disease of  
Poultry, 8th ed., 270-278 (1984)). Three components present on the cell surface of  
*E. coli* assist in promoting the survival of *E. coli* from serum and phagocytosis  
25   (Timmis *et al.*, Current Topics in Micro. and Immunol., 118:197-218 (1985)).  
These three components are the acidic polysaccharide capsules, O-antigen  
lipopolysaccharide ("O-antigens") and outer membrane proteins ("OMPs"). Some

phagocytes, are involved in phagocytosis and include such cells as blood monocytes, neutrophils and tissue macrophages.

Complement resistance of *E. coli* has generally been reported as related to several potential structural factors including a K1-antigenic capsule (Aqüero *et al.*, *Infect. Immun.*, 40:359-368 (1984)), or other capsule type (Russo *et al.*, *Infect. Immun.*, 61:3578-3582 (1993)), a smooth lipopolysaccharide (LPS) layer (Cross *et al.*, In: *Bacteria, Complement and the Phagocytic Cell*, Vol. H24, F. C. Cabello and C. Pruzzo, eds., Springer-Verlag, Berlin, pp. 319-334 (1988)), and certain OMPs including TraT (Montenegro *et al.*, *J. Gen. Microbiol.* 131:1511-1521 (1985); Moll *et al.*, *Infect. Immun.* 28:359-367 (1980)), Iss (Binns *et al.*, *Infect. Immun.*, 35:654-659 (1982); Chuba *et al.*, *Mol. Gen. Genet.*, 216:287-192 (1989)), and OmpA (Weiser *et al.*, *Infect. Immun.* 59:2252-2258 (1991)). The absence of capsule as a complement-resistance mechanism in disease-associated avian *E. coli* isolates suggests that such isolates must employ other means to avoid the killing effects of complement.

Iss is an OMP encoded by an avian *E. coli iss* gene. Some reports have indicated that *E. coli* isolates from avian subjects with a septicemic disease are much more likely to have *iss*-related sequences than are *E. coli* isolates of apparently healthy birds. However, no significant difference in the distribution of *traT*-related sequences (*traT* encodes the OMP TraT) has been found in the same isolates. The plasmid location of *iss* and *traT* suggests that their presence in different isolates might be more variable than the chromosomally-located *ompA* gene (Weiser *et al.*, *Infect. Immun.*, 59:2252-2258 (1991)).

The expression of a human *E. coli iss* gene was found to increase the virulence of *E. coli* containing the gene by 100-fold for one-day old chicks and to increase the complement resistance of transformed cells over 20-fold for one-day old chicks (Binns *et al.*, *Infect. Immun.*, 35:654-659 (1982)). Genetic evidence suggests that *iss* obtained from human *E. coli* is a derivative of a lambda gene known as *bor* (Barondess *et al.*, *J. Bacteriol.*, 177:1247-1253 (1995); Barondess *et*

*al.*, Nature, 346:871-874; (1990); Chuba *et al.*, Mol. Gen. Genet., 216:287-292 (1989)). Bor is a lipoprotein of the cell envelope of *E. coli* lambda lysogens and appears to confer serum resistance on these lysogens (Barondess *et al.*, J. Bacteriol., 177:1247-1253 (1995)). The high sequence identity between the Bor polypeptide  
5 and an avian Iss polypeptide suggests that Iss is also targeted to the outer membrane.

Septicemic colibacillosis is an economically devastating problem for the poultry industry in the United States. It is the most costly bacterial disease of production poultry animals causing multi-million dollar losses by the poultry industry annually. Control of this disease is hampered by the lack of a basic  
10 understanding about the virulence mechanisms employed by avian *E. coli*. For example, no single identifiable virulence marker has been associated with disease-causing avian *E. coli*. In other animal species, such as cattle, certain markers of virulence have been identified, and these markers have been used to facilitate epidemiologic studies and to develop control strategies designed to decrease the  
15 detrimental impact of colibacillosis on animal agriculture (Butler *et al.*, In: "Escherichia coli in Domestic Animals and Humans," G.L. Gyles (ed.), CAB International, Wallingford, UK:91-116 (1994)).

Therefore, a need exists to identify genes and other markers associated with complement-resistance of *E. coli* in birds. These genes can function as markers for  
20 disease-causing avian *E. coli* and the detection of these genes may form the basis for improved diagnostic and control strategies for avian colibacillosis, in addition to the formulation and preparation of immunogenic compositions useful to prevent or inhibit avian septicemic diseases.

## 25 Summary of the Invention

*iss* has been identified as a marker for avian *E. coli* virulence and complement-resistance in animals. *E. coli* is associated with septicemic disease in birds, bovine colibacillosis, including scours and septicemic disease, in cattle, and septicemic disease in other animals. As used herein, the term *iss* or *iss* "gene" refers

to a nucleic acid sequence or molecule that encodes an avian *E. coli* *Iss* polypeptide. It is to be understood that, unless otherwise indicated, the term "nucleic acid sequence" includes a nucleic acid sequence as well as the complementary nucleic acid sequence derived from that nucleic acid sequence. The present invention  
5 provides an isolated and purified nucleic acid molecule that encodes an avian *E. coli* *Iss* polypeptide, a biologically active variant or subunit thereof. As described more fully below, an "Iss polypeptide" also includes an Iss "fusion" polypeptide.

A biologically active Iss polypeptide, variant or subunit thereof falling within the scope of the invention has at least about 1%, preferably at least about 10%, more  
10 preferably at least about 50%, and even more preferably at least about 90%, the activity of the polypeptide comprising SEQ ID NO:2. A preferred nucleic acid sequence is a DNA molecule encoding an Iss polypeptide (SEQ ID NO:2).

Another preferred embodiment of the invention is an isolated nucleic acid molecule including nucleotides 73-309 of SEQ ID NO:22, more preferably, further  
15 including nucleotides 1-33 of SEQ ID NO:21, located 5' of nucleotides 73-309 of SEQ ID NO:22. Biological samples from a subject suspected of being exposed to, a carrier of, or afflicted with a septicemic disease, such as colibacillosis, can be analyzed for the presence of an *iss* nucleic acid sequence or an Iss polypeptide sequence encoded thereby. As used herein, the term "septicemia" or "septicemic  
20 disease" includes, but is not limited to, several avian and non-avian diseases such as air-sacculitis, pneumonitis, septicemic colibacillosis, colisepticemia, cellulitis, and scours.

The present invention further provides an expression cassette comprising a preselected *iss* nucleic acid sequence operably linked to a promoter and functional in  
25 a host cell wherein said nucleic acid sequence encodes an Iss polypeptide, a biologically active variant or subunit thereof. The expression cassettes can be placed into expression vectors. These recombinant vectors can then be employed to transform prokaryotic and eukaryotic host cells. The expression of the preselected nucleic acid sequence in the transformed cell results in the production of

recombinant Iss polypeptide or an Iss fusion polypeptide depending on the selected vector. As used herein, an Iss "fusion" polypeptide or protein is a product of a first preselected nucleic acid sequence, for example a preselected *iss* sequence, and a second nucleic acid sequence, for example a glutathione S-transferase sequence, operably linked at either the carboxyl terminal or amino terminal of the first sequence. The expression of this chimeric gene results in a single or continuous polypeptide or protein when expressed and isolated from a host cell. The product of this expression can enhance properties relating to, for example, purification, isolation, targeting and increased immunogenicity.

10           The present invention further provides a method for detecting an avian *E. coli iss* nucleic acid sequence encoding an Iss polypeptide. The method includes contacting a sample containing nucleic acids with a first and a second oligonucleotide, under conditions effective to amplify at least one nucleic acid to yield an amplified nucleic acid molecule. The amplified nucleic acid molecule  
15 includes at least a portion of the nucleic acid sequence encoding an avian *E. coli* Iss polypeptide. The method further includes detecting the presence or absence of the amplified nucleic acid molecule, wherein the presence of the amplified nucleic acid indicates the presence of an avian *E. coli iss* nucleic acid sequence encoding an Iss polypeptide. The amplified nucleic acid molecule can further include the nucleic  
20 acid sequence encoding an avian *E. coli* Iss polypeptide. The method can also be used to determine whether a subject is infected with an avian pathogenic *E. coli*.

          The first oligonucleotide can hybridize to nucleic acid sequences that are present 5' of the nucleic acid sequence encoding an avian *E. coli* Iss polypeptide. For instance, the nucleic acid sequences to which the first oligonucleotide hybridize  
25 can include at least 13 nucleotides of nucleotides 1 to 291 of SEQ ID NO:1.

          The second oligonucleotide can hybridize to nucleic acid sequences that are present 3' of the nucleic acid sequence encoding an avian *E. coli* Iss polypeptide. For instance, the nucleic acid sequences to which the first oligonucleotide hybridize comprise at least 13 nucleotides of nucleotides 601-760 of SEQ ID NO:1.

Alternatively, the first and/or second oligonucleotide can hybridize to nucleic acid sequences that encode a portion of an avian *E. coli* Iss polypeptide. For instance, the avian *E. coli iss* nucleic acid sequence to which at least one oligonucleotide hybridizes can include at least 13 nucleotides of SEQ ID NO:22.

5           Techniques of nucleic acid detection include DNA and RNA amplification methods and includes such methods as polymerase chain reaction ("PCR"). Additionally, the detection of antibody responses specific for an Iss polypeptide encoded by an *iss* sequence can be used, for example, in ELISA-based immunoassays for the serodiagnosis of a subject suspected of being exposed to, a  
10   carrier of, or afflicted with a septicemic disease. The presence or amount of an *iss* or an Iss sequence in a sample can then be compared to a control sample, *e.g.*, an subject known to be disease free.

Nucleic acid sequences of the invention can be produced by nucleic acid amplification techniques using novel oligonucleotide primers, such as, SEQ ID  
15   NOS: 3, 4, 11, 12, 13, 14 and 15 employed in the synthesis. These novel primers can also used as probes and in PCR to detect other homologous *iss* nucleic acid sequences. An oligonucleotide or primer of the invention preferably has at least about 70%, more preferably at least about 85%, and most preferably at least about 90%, sequence identity or homology to SEQ ID NO:1.

20           The present invention also provides a polypeptide that can be used as a capture antigen to bind anti-polypeptide or anti-Iss antibodies in a biological sample obtained from a subject. For example, a biological sample comprising antibodies can be mixed with a purified Iss polypeptide to yield an antigen-antibody complex. The antibodies that are bound to the antigen are separated from the antibodies that  
25   are not bound to the antigen. The antigen-antibody complex is then detected or determined.

The invention further provides a method for detecting or determining the presence of Iss antibodies in a biological sample obtained from a subject. The method comprises contacting an amount of a purified Iss polypeptide, variant or

subunit thereof with the biological sample that is suspected of comprising antibodies specific for the polypeptide for a sufficient time to form complexes between at least a portion of the antibodies and a portion of the purified polypeptide. The presence or amount of the complexes is then determined or detected.

5           In another embodiment of the present invention, monoclonal and polyclonal antibodies directed against an avian *E. coli* Iss polypeptide or fusion polypeptide are prepared. The recombinant polypeptide and antibodies are useful in the development of assays to detect Iss-producing *E. coli*.

          In yet another embodiment of the invention, an avian Iss polypeptide, variant  
10 or subunit thereof can be used to produce populations of antibodies that, in turn can be used as the basis for assays to detect and quantify Iss polypeptide (or "protein") in samples derived from a subject. Also provided are populations of monoclonal and polyclonal antibodies that specifically bind to an avian *E. coli* Iss polypeptide or GST-Iss fusion protein. These antibodies can also be used in affinity  
15 chromatography, to purify avian Iss from natural sources.

          Also provided is a diagnostic kit for detecting or determining antibodies that specifically react to an avian *E. coli* Iss polypeptide, variant or subunit thereof. The kit comprises packaging, containing, separately packaged, a solid phase capable of binding a polypeptide and a known amount of a purified Iss polypeptide. Preferably,  
20 the polypeptide has an amino acid sequence comprising SEQ ID NO:2, a biologically active variant or biologically active subunit thereof.

          In another embodiment, an isolated and purified avian *E. coli* Iss polypeptide can be employed as a component in a diagnostic assay for "native" Iss in samples derived from biological samples. For example, polypeptide can be bound to a  
25 detectable label and employed in a competitive immunoassay for Iss protein.

          Another preferred embodiment of the invention provides an immunogenic composition or a vaccine comprising an isolated and purified avian *E. coli* Iss polypeptide, or a nucleic acid molecule encoding an avian *E. coli* Iss polypeptide, wherein the administration of the immunogenic composition or vaccine to a subject

induces the production of antibodies to said polypeptide. The invention further includes a method of treating an animal at risk of exposure to or afflicted with, a pathogenic *E. coli* that includes administration of the immunogenic composition or vaccine to the animal. The produced antibodies can inhibit or block subsequent  
5 infection of the host by a complement resistant and/or virulent avian *E. coli*. Preferably, the immunogenic composition or a vaccine is administered in combination with a pharmaceutically acceptable carrier.

### **Brief Description of the Figures**

10 Figure 1 shows partial alignment of the DNA sequence of the *iss* gene obtained from a virulent, complement-resistant avian *E. coli* isolate (102iss; SEQ ID NO:22), the DNA sequence of an *iss* gene from a septicemic human *E. coli* isolate (Eciss; SEQ ID NO:5), and the DNA sequence of the lambda *bor* gene (lambor; SEQ ID NO:6). The vertical line between nucleotides of each sequence indicate  
15 identity.

Figure 2 shows alignment of the predicted amino acid sequence of Iss from a virulent, complement-resistant avian *E. coli* (102Iss; SEQ ID NO:2); Iss from a septicemic human *E. coli* isolate (Iss\_Ec; SEQ ID NO:7), and the lambda Bor protein (lamBor; SEQ ID NO:8). The vertical line between amino acids of each  
20 sequence indicate identity; the colon indicates a conserved amino acid.

Figure 3 shows the *iss* gene sequence (SEQ ID NO:22) cloned in frame into expression vector pGEX-6P-3, including the PRESCISSION protease cleavage site (SEQ ID NO:21). The polypeptide encoded by SEQ ID NO:21, designated SEQ ID NO:20, is also shown. The GST fusion sequence, not shown, is located upstream of  
25 the PRESCISSION protease cleavage site. The amplified *iss* gene was cloned into the *Bam*HI and the *Eco*RI sites of the pGEX-6P-3 vector.

Figure 4 shows an SDS-PAGE of crude total protein preparations of a protease-deficient *E. coli* containing the expression vector, pGEX-6P-3, or pGEX-6P-3 comprising the *iss* gene sequence, in the "uninduced" or "induced" state. Lane



S = molecular weight standard in kD (Pharmacia Biotech Inc., Piscataway, N.J.); Lane 1U = pGEX-6P-3 alone in the "uninduced" state; Lane 3U = pGEX-6P-3 comprising *iss* in the "uninduced" state; Lane 1I = pGEX-6P-3 alone in the "induced" state; and lane 3I = pGEX-6P-3 comprising *iss* in the "induced" state.

- 5 Note that lane 3I shows a 37 kD protein band not present in lanes 1U, 3U or 1I which is consistent with an GST-Iss fusion polypeptide product.

Figure 5 shows the crude total protein preparation of a protease-deficient *E. coli*, containing pGEX-6P-3 comprising the *iss* gene sequence. Total protein was prepared 3 hours post-induction of expression with IPTG. After resolution by SDS-  
10 PAGE, the proteins were transferred to PVDF and the blot probed with anti-GST. Lane S = molecular weight standard in kD; Lanes 1, 2 and 3 = contain the same amount of crude bacterial lysate but different concentrations of anti-GST (*e.g.*, base solution of 5/mg/ml diluted to 1:500 (lane 3), 1:1000 (lane 2) and 1:2000 (lane 1)). In a second PVDF blot using crude lysates of uninduced bacteria that was probed in  
15 a technique as described above, no bands were recognized (not shown), thus confirming the 37-kD band present in induced bacteria is the GST-Iss fusion protein.

Figure 6 shows a diagram of the *iss*-pVAX1 clone (pSH551). PMB1 ori, origin of replication; Pcmv, human cytomegalovirus immediate-early promoter/enhancer; *iss*, coding sequence insert; BGH polyA, bovine growth  
20 hormone polyadenylation signal; kanamycin, kanamycin resistance coding sequence.

### **Detailed Description of the Invention**

The identification and association of *iss* and Iss with clinical disease, *e.g.*, septicemia, in birds due to virulent and complement resistant *E. coli* has been  
25 established. The data obtained from the isolation and characterization of *iss* and Iss sequences has clarified the disease-causing potential of isolates that carry the *iss* gene, and established the importance of Iss to avian *E. coli* virulence and complement resistance. Moreover, *iss* and Iss employed as markers enable the development of diagnostic procedures for use in avian colibacillosis outbreaks and

epidemiological studies and provide development of such diagnostic procedures by production of, for example, monoclonal antibodies to Iss.

### **Definitions**

5 In describing the present invention, the following terminology will be used in accordance with the definitions set out below.

The terms "isolated and/or purified," refer to *in vitro* preparation, isolation and/or purification of a nucleic acid molecule, polypeptide or peptide of the invention, so that it is not associated with *in vivo* substances. It is to be understood  
10 that an isolated and/or purified nucleic acid molecule, polypeptide, or peptide can be a synthetic or recombinant molecule as well as one actually isolated from its native cellular environment.

As used herein, an "Iss polypeptide or amino acid sequence" is preferably an avian *E. coli iss* derived polypeptide having an amino acid sequence comprising  
15 SEQ ID NO:2, or a biologically active variant or subunit thereof. An avian *E. coli* derived Iss polypeptide can be naturally occurring, synthetic, or recombinant. A "variant" Iss polypeptide is a polypeptide having an amino acid sequence that has at least about 87%, preferably at least about 90%, and more preferably at least about 95%, but less than 100%, sequence identity or homology to SEQ ID NO: 2 or a  
20 biologically active subunit thereof. A variant polypeptide, peptide or amino acid sequence of the invention may include amino acid residues not present in the corresponding wild type Iss polypeptide or peptide, as well as internal deletions relative to the corresponding wild type Iss polypeptide. As used herein, a "subunit" is a biologically active portion, region or peptide of a full-length Iss polypeptide,  
25 *e.g.*, SEQ ID NO:2. A subunit of an avian *E. coli* Iss derived polypeptide preferably has at least 7 amino acids, more preferably at least 10 amino acids, most preferably at least 14 amino acids.

The percent sequence identity between a "variant" Iss polypeptide and a full-length Iss polypeptide, *e.g.*, SEQ ID NO:2, is generally determined by aligning the

residues of the two amino acid sequences (i.e., a candidate amino acid sequence and the amino acid sequence of SEQ ID NO:2) to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. A candidate amino acid sequence is the amino acid sequence being compared to an amino acid sequence present in SEQ ID NO:2. A candidate amino acid sequence can be isolated from an avian *E. coli*, or can be produced using recombinant techniques, or chemically or enzymatically synthesized. Preferably, two amino acid sequences are compared using the Blastp program, version 2.1.2, of the BLAST 2 search algorithm, as described by Tatusova, et al. (*FEMS Microbiol Lett* 1999, 174:247-250), and available at [www.ncbi.nlm.nih.gov/gorf/bl2.html](http://www.ncbi.nlm.nih.gov/gorf/bl2.html). Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x\_dropoff = 50, expect = 10, wordsize = 3, and filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, percent identity is referred to as "identities."

Preferably, the polypeptides and peptides of the instant invention are biologically active. For example, biologically active Iss polypeptides, peptides and variants thereof falling within the scope of the invention have at least about 1%, preferably at least about 10%, more preferably at least about 50%, and even more preferably at least about 90%, the activity of the polypeptide comprising SEQ ID NO:2. The activity of an Iss polypeptide or peptide, can be measured by methods well known to the art including, but not limited to, the ability of the polypeptide or peptide to be bound by antibodies specific for Iss (e.g., specific for the Iss protein) or the ability of the polypeptide or peptide to elicit a sequence-specific immunologic response when the polypeptide is administered to animals such as birds, cattle, or mink. An avian *E. coli* Iss polypeptide, peptide, variant, subunit or fragment thereof is "immunologically reactive" with an antibody when it binds to an antibody due to

antibody recognition of a specific epitope contained within the polypeptide.

Immunological reactivity may be determined by antibody binding, more particularly by the kinetics of antibody binding, and/or by competition in binding using as competitor(s) a known polypeptide(s) containing an epitope against which the antibody is directed. The techniques for determining whether a polypeptide is immunologically reactive with an antibody are known in the art.

Preferably, the immunologic response is a humoral response, *i.e.*, antibody response, directed to a particular epitope on the polypeptide or peptide. More preferably, the presence of antibodies specific for an *Iss* epitope correlates with a septicemic infection in a subject. As used herein, "epitope" refers to an antigenic determinant of a polypeptide. An epitope could comprise 3 amino acids in a spatial conformation which is unique to the epitope. Generally an epitope consists of at least 5 such amino acids, and more usually, consists of at least 8-10 such amino acids. Methods of determining the spatial conformation of amino acids are known in the art, and include, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance.

An isolated "variant" nucleic acid sequence of the present invention is a nucleic acid sequence that has at least 87%, preferably at least about 90%, and most preferably at least about 95%, but less than 100%, nucleic acid sequence identity or homology to a the nucleotide sequence of the corresponding wild type nucleic acid molecule, *e.g.*, a DNA sequence comprising SEQ ID NO:22. However, a variant nucleic acid molecule of the invention may include nucleotide bases not present in the corresponding wild type nucleic acid molecule, as well as internal deletions relative to the corresponding wild type nucleic acid molecule. As used herein a nucleic acid "subunit" is a biologically active portion or region of a full-length *iss* nucleic acid sequence, *e.g.*, SEQ ID NO:22, or a portion or region of an *iss* nucleic acid sequence that encodes a biologically active subunit of an *Iss* polypeptide. The percent identity between a "variant" nucleic acid sequence of the present invention and a wild type nucleic acid molecule, *e.g.*, SEQ ID NO:22, is determined by

aligning the residues of the two polynucleotides (i.e., the candidate nucleotide sequence and the nucleotide sequence of the coding region of SEQ ID NO:22) to optimize the number of identical nucleotides along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to

5 optimize the number of shared nucleotides, although the nucleotides in each sequence must nonetheless remain in their proper order. A candidate nucleotide sequence is the nucleotide sequence being compared to SEQ ID NO:22. A candidate nucleotide sequence can be isolated from an *E. coli*, preferably an *E. coli* obtained from a bird, or can be produced using recombinant techniques, or

10 chemically or enzymatically synthesized. Preferably, two nucleotide sequences are compared using the Blastn program, version 2.1.2, of the BLAST 2 search algorithm, as described by Tatusova, et al. (*FEMS Microbiol Lett* 1999, 174:247-250), and available at [www.ncbi.nlm.nih.gov/gorf/bl2.html](http://www.ncbi.nlm.nih.gov/gorf/bl2.html). Preferably, the default values for all BLAST 2 search parameters are used, including reward for

15 match = 1, penalty for mismatch = -2, open gap penalty = 5, extension gap penalty = 2, gap x\_dropoff = 50, expect = 10, wordsize = 11, and filter on. In the comparison of two nucleotide sequences using the BLAST search algorithm, percent identity.

The term "hybridization under stringent conditions" refers to those conditions that (1) employ low ionic strength and high temperature for washing, for

20 example, 0.015 M NaCl/0.0015 M sodium citrate (SSC); 0.1% sodium lauryl sulfate at about 68° C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C. Another example is use

25 of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42° C, with washes at 42° C in 0.2 x SSC and 0.1% SDS.

As used herein, the term "target region" refers to a region of the nucleic acid

which is to be amplified and/or detected.

A "genomic clone" refers to a DNA fragment derived directly from cellular DNA rather than from messenger RNA.

Unless otherwise specified, the terms "a," "an," "the," and "at least one" are  
5 used interchangeably and mean one or more than one.

## **Background**

The general techniques used in cloning avian *E. coli* iss nucleic acid sequences (*e.g.*, PCR technology, sequencing nucleic acid clones and polypeptides  
10 derived therefrom, constructing expression vectors or expression cassettes, transforming cells, performing immunological assays such as radioimmunoassays and ELISA assays, and growing cells in culture) are known in the art and laboratory manuals are available describing these techniques. *See*, for example, Sambrook,  
Fitsch & Maniatis, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor  
15 Laboratory Press (1989); DNA Cloning, Volumes I and II (D. N. Glover ed. 1985); Oligonucleotide Synthesis (M. J. Gait ed, 1984); Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Animal Cell Culture (R. I. Freshney ed. 1986); Immobilized Cells and Enzymes (IRL Press, 1986); B. Perbal; A Practical Guide to Molecular Cloning (1984); the Series, Methods in Enzymology (Academic Press,  
20 Inc.); Immunochemical Methods in Cell and Molecular Biology (Academic Press, London), Scopes, (1987), Protein Purification: Principles and Practice, Second Edition (Springer-Verlag, N.Y.), and Handbook of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell eds 1986).

## **I. Identification of Isolates Falling Within the Scope of the Invention**

### **A. Sources of Avian *E. coli* Isolates**

*E. coli* isolates have been identified a variety of avian subjects (*e.g.*, turkeys, chickens and ducks) clinically diagnosed with a septicemic disease or exposed to other avians having, afflicted with, or suspected of a septicemic disease. Biological

or environmental samples can be assayed to determine whether they contain an *E. coli* isolate. A biological sample is one that is derived from, obtained from, or collected from an animal. A biological sample can be obtained from any animal suspected of harboring pathogenic a pathogenic *E. coli* that expresses an avian *E.*

- 5 *coli* *iss* polypeptide or variant or subunit thereof including, without limitation, farm animals such as poultry, cattle and mink. Preferably, the biological sample is from an avian subject, for instance, a turkey, a chicken, or a duck. Biological samples can be obtained, for example, from tissues, organs, blood, serum, bone and yolk sacs of the animal. Biological samples can also be obtained from eggs, joints, excretions, or  
10 external or internal lesions such as those found on wattle, skin, subcutaneous tissues, heart, liver, brain, air sac, lung, or oviduct, without limitation. Environmental samples can be obtained, for instance, from the litter, feed, or water present in an area used to house an animal.

- A phenotypic comparison of 40 avian *E. coli iss* isolates from the intestines  
15 of normal chickens and 40 avian *E. coli iss* isolates from the organs of colisepticemic chickens was performed. The isolates from colisepticemic chickens were shown to be more likely to produce siderophores and type I pili, and exhibit greater complement resistance, than were the intestinal isolates from normal chickens. A closer examination of the 10 most complement-resistant colisepticemic  
20 *iss* isolates and the 10 most complement-sensitive intestinal *iss* isolates revealed that an *iss* isolate's ability to resist complement directly correlated with an isolates ability to kill chick embryos (Wooley *et al.*, *Avian Dis.*, 36:679-684 (1992)).

- The lytic activity of complement on test isolates was determined by a quantitative microtiter test method (Lee *et al.*, *Avian Dis.* 35:892-896 (1991)). The  
25 quantitative microtiter test results were analyzed by calculating the regression coefficients ( $\beta$ ) of the slopes of growth over time. The  $\beta$  values were ranked and compared using a Dunnett's test ( $\alpha = 0.05$ ). These rankings permitted the classification an *E. coli* as either sensitive, intermediate or resistant to the action of chicken complement. This observation was confirmed by mutational analysis.

Specifically, a mutant *E. coli* differing from a virulent wild-type *E. coli* in the single factor of complement resistance was prepared and tested (Nolan *et al.*, Avian Dis., 36:398-402 (1992)). Characterization of this complement-sensitive mutant revealed that it was less virulent than the wild-type isolate (Kottom *et al.*, Avian Dis. (1997);

5 Nolan *et al.*, Avian Dis., 38:146-150 (1994); Nolan *et al.*, Avian Dis., 36:398-402 (1992)). This attribute provides further evidence that complement resistance is an important contributor in the observed virulence of avian *E. coli*.

The occurrence of *iss* related sequences among clinical human and avian *E. coli* isolates is very informative. For example, Fernandez-Beros *et al.*, J. Clin.

10 Micro., 28:742-746 (1990) examined 200 *E. coli* isolates from patients with diarrhea and 146 isolates from patients with bacteremia for their possession of several virulence-related sequences including *traT* and *iss*. No difference was found between the two groups in distribution of these two genes. The *iss*-related sequences occurred in about 25-28% of all isolates examined, and the *traT*-related

15 sequences occurred in 41- 44% of these same isolates.

In the present invention, 210 isolates, derived from a variety of sources, such as tissue, bone, blood, serum and yolk sacs of birds with colibacillosis and 56 fecal isolates of healthy birds were examined. It was found that 76% of the isolates of sick chickens contained *iss*-related sequences, and only 23% of the isolates of

20 healthy chickens contained such sequences. There was no significant difference in the distribution of *traT*-related sequences between the two groups, suggesting that *traT* was not an important virulence factor for avian *E. coli* isolates. Conversely, the large difference in the distribution of *iss* among isolates of birds suggests *iss* was a potentially important virulence factor for avian *E. coli*. Additionally, the difference

25 in distribution of *iss*-related sequences in isolates from birds and humans with extraintestinal colibacillosis suggested that *iss* was potentially more important for complement resistance and virulence with avian *E. coli* than with human *E. coli*.

The differences in the distribution of *iss* and capsule, a polysaccharide layer located outside the cell wall of bacteria, among avian and human *E. coli* isolates



further emphasize the intriguing differences between avian and mammalian colibacillosis and suggest that the avian model of colibacillosis is a unique system in which to observe complement resistance. The uniqueness of this system reflects the nature of colibacillosis in birds, the entry point of *E. coli* into a host bird, and the initial sites of colonization of the host. As mentioned previously, the route of entry for most forms of avian colibacillosis is the respiratory tract. Enteric forms of avian colibacillosis are rare (Gyles, In: Pathogenesis of Bacterial Infections in Animals, 2nd ed., C. L. Gyles and C. O. Thoen, eds., Iowa State University Press, Ames, IA, pp. 164-187 (1993)). This is not the case in mammals, where intestinal forms of colibacillosis are common (Gyles, In: Pathogenesis of Bacterial Infections in Animals, 2nd ed., C. L. Gyles and C. O. Thoen, eds., Iowa State University Press, Ames, IA, pp. 164-187 (1993)). Moreover, the entry point taken by most disease-causing *E. coli* of avians is primarily through the respiratory tract, unlike mammals, wherein the route of initial entry is likely to be the gastrointestinal tract (Gyles, In: Pathogenesis of Bacterial Infections in Animals, 2nd ed., C. L. Gyles and C. O. Thoen, eds., Iowa State University Press, Ames, IA, pp. 164-187 (1993)).

It is known to the art that while most of the serotypes or *E. coli* isolated from poultry are pathogenic only for birds, a few are also associated with disease conditions in other animals (Gross, Colibacillosis, In: Diseases of Poultry, Hofstad et al., (eds.), The Iowa State University Press, pp. 270-279 (1984)). For instance, serotype O78:K80 is commonly isolated from cattle and sheep. Moreover, serotypes of *E. coli* frequently isolated from septicemic poultry are not among those pathogenic for humans. Since *E. coli* causing infections in birds can also be found in other animals, the term "avian *E. coli* iss nucleic acid sequence," as used herein, refers to nucleic acid sequences that are present in *E. coli* that may be present and pathogenic in birds as well as other animals. Nucleic acid sequences encoding avian *E. coli* Iss polypeptides can therefore be derived from and/or detected in nonavian as well as avian sources. For example, a nucleic acid sequence encoding avian *E. coli* Iss polypeptide can be derived from or detected in a farm animal known or believed

to be naturally or experimentally infected by a virulent, septicemia-causing avian *E. coli*, including, for instance, poultry, cattle, or mink.

#### **B. Isolation of Nucleic Acid Molecules of the Invention**

- 5           A nucleic acid molecule encoding an *Iss* polypeptide can be identified and isolated using standard methods, as described by Sambrook *et al.*, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989). For example, polymerase chain reaction can be employed to isolate and clone *iss* genes. "Polymerase chain reaction" or "PCR" refers to a procedure or technique wherein
- 10       amounts of a preselected piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite or complimentary strands of the template to be amplified. PCR can be used to amplify
- 15       specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences and the like, to yield an amplification product. *See also*, Mullis *et al.*, Cold Harbor Symp. Quant. Biol., 51:263 (1987); Erlich, ed., PCR Technology (Stockton Press, NY, 1989).
- 20           *iss* nucleic acid sequences can be isolated from septicemic tissue samples by PCR techniques employing *iss* specific oligonucleotide primers, for example, SEQ ID NOs: 3, 4, 11, 12, 13, 14, and 15. An oligonucleotide or oligonucleotide pair is specific for an avian *E. coli iss* nucleotide sequence when it amplifies a DNA fragment that includes at least a portion of a nucleotide sequence encoding avian *E.*
- 25       *coli Iss* polypeptide, and the oligonucleotide or oligonucleotide pair does not amplify a nucleotide sequence encoding *Iss* that is present in an *E. coli* causing infections in humans, or is present in  $\lambda$  bor. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. Primers can be made to correspond to nucleic acid molecules encoding highly conserved regions

of polypeptides. Preferably, at least one primer used in an amplification reaction, more preferably, both primers, corresponds to nucleotide sequences that are present 5' (i.e., upstream) or 3' (i.e., downstream) of a nucleotide sequence encoding an avian *E. coli* Iss polypeptide; i.e., nucleotide sequences that "flank" the coding region for avian *E. coli* Iss polypeptide. Preferably, a primer is at least about 13 nucleotides, more preferably at least about 17 nucleotides, most preferably at least about 20 nucleotides in length. Preferably, a primer is no greater than about 27 nucleotides, more preferably no greater than about 25 nucleotides, most preferably no greater than about 22 nucleotides in length. However, these nucleic acid molecules can be introduced into expression cassettes, which, when expressed in a host, can provide "anti-sense" nucleic acid transcripts. Thus, the present invention also provides isolated and purified "antisense" nucleic acid molecules that have at least about 80%, preferably about 90%, and most preferably at least about 98%, nucleotide sequence complementary to SEQ ID NOs. 1, 11, 12, 13, 14 and 15.

Alternatively, nucleotide sequences can be obtained and prepared by a sequence comparison of other related genes, such as the *traT* and *ompA* sequences. Preferably, at least two primers are prepared, one of which is predicted to anneal to the antisense strand, and the other of which is predicted to anneal to the sense strand of a nucleic acid molecule that encodes the avian *E. coli* Iss polypeptide. The products of each PCR reaction are separated by agarose gel electrophoresis, and the consistently amplified products are purified and cloned directly into a suitable vector such as a plasmid vector. Products obtained therefrom can be sequenced manually using standard procedures or with an automated sequencer, for example, LICOR.

Alternatively, DNA libraries may be probed using the procedure of Grunstein and Hogness Proc. Natl. Acad. Sci. USA, 73:3961 (1975), or other available techniques as described in Sambrook *et al.*, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989). Briefly, in this procedure, the DNA to be probed is immobilized on a membrane (*e.g.*, nitrocellulose or nylon filters) denatured, and prehybridized with a buffer containing

0-50% formamide, 0.75 M NaCl, 75 mM Na citrate, 0.02 % (wt/v) each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll, 50 mM Na phosphate (pH 6.5), 0.1% SDS, and 100 micrograms/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps depends on the stringency required. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and/or longer hybridization times. Both non-radioactive and radioactive techniques can be utilized. Probes containing more than 30 or 40 nucleotides such as those derived from genomic sequences generally employ higher temperatures, *e.g.*, about 40° - 42°C, and a high percentage, *e.g.*, 50%, formamide. Following prehybridization, 5'-<sup>32</sup>P-labeled oligonucleotide probe is added to the buffer, and the filters are incubated in this mixture under hybridization conditions. After washing, the treated filters are subjected to autoradiography or a non-radioactive technique such as DIGOXIGENIN D/UTP labeling kit (Boehringer Mannheim, Indianapolis, Ind.), to show the location of the hybridized probe; DNA in corresponding locations on the original agar plates is used as the source of the desired DNA.

The nucleotide sequence of an avian *E. coli iss* gene has about a 96.8% sequence identity in a 310 bp overlap, as shown in Figure 1, when compared to the entire sequence of a human *E. coli iss* gene (Chuba *et al.*, Mol. Gen. Genet., 216:287-292 (1989)). Surprisingly, the avian *E. coli iss* gene has an insertion at nucleotide 158 and a deletion at nucleotide 189, that results in a frameshift in the resulting polypeptide sequence (SEQ ID NO:2). The 10 amino acid change located in the middle of the protein creates an approximate 86.3 % sequence identity in a 102 amino acid overlap, as shown in Figure 2, between the human ("Iss\_Ec") and avian ("102Iss") *E. coli* Iss polypeptides. The 102Iss amino acid sequence and the Iss\_Ec amino acid sequence are about 86.3% identical in a 102 amino acid overlap. The low identity between these two *E. coli* Iss sequences is due to a frameshift that has occurred in Iss\_Ec. The amino-terminal region of 102Iss has structural features

characteristic of a cleavable signal sequence and spans amino acids 17 to 22.

Residues 17-21 of Iss are almost identical to residues 12-17 of Bor (Figure 2).

When the avian *E. coli iss* sequence was examined with Gene Inspector software (Textco, Inc., West Lebanon, New Hampshire), it was found that the avian  
5 *E. coli* Iss protein is predicted to have an isoelectric point of approximately 8.47, and at pH 7, is expected to have a net charge of +2.05. An Iss polypeptide is predicted to be approximately a 10-11 kD protein containing 102 amino acids that is resistant to acid hydrolysis. Additionally, based on the Iss polypeptide's predicted folding characteristics and hydropathy plots, Iss is likely to have a number of  
10 accessible sites, for example, sites not buried in the bacterial membrane, that are antigenic.

The *iss* locus of the conjugative plasmid ColV, I-K94, is closely related to the phage lambda *bor* gene. Sequencing has shown that *iss* displays a discrete 796 bp block of DNA homologous to a region from bp 46186 to 46982 in the lambda  
15 sequence (Chuba *et al.*, *Mol. Gen. Genet.*, 216:287-292 (1989); Sanger *et al.*, *J. Mol. Biol.*, 162:729-773 (1982)). The overall identity between lambda *bor* and *iss* is about 81%, excluding gaps, as the avian *E. coli iss* nucleotide sequence has an approximate 89.4% sequence identity in a 303 bp overlap with the lambda *bor* gene (Barondess *et al.*, *J. Bacteriol.*, 177:1247-1253 (1995); Barondess *et al.*, *Nature*,  
20 346:871-874 (1990)). The 102Iss amino acid sequence and the lamBor amino acid sequence are about 89.7% identical in a 97 amino acid overlap.

### **C. Variants of the Nucleic Acid Molecules of the Invention**

Nucleic acid molecules encoding amino acid sequence variants of an Iss  
25 polypeptide can be prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and

cassette mutagenesis of an earlier prepared variant or a non-variant version of an Iss polypeptide.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing amino acid substitution variants of an Iss polypeptide. This technique is well known in the art as described by Adelman *et al.*, DNA 2:183 (1983), and Zoller Nucleic Acids Res., 10:6487 (1982). Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified and having the desired modification included in its own sequence. The resulting double-stranded DNA is transformed into a phage supporting host bacterium. As used herein, a "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine or cytosine) in its normal, double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids and chromosomes. In discussing the structure of a particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA). Cultures of the transformed bacteria, which contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically, 50% of the new plaques contain phage having the mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to a labeled synthetic probe at temperatures and conditions that permit hybridization with the correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization are recovered and then cloned.

A DNA sequence encoding an Iss polypeptide can be synthetically prepared rather than isolated. The DNA sequence can be designed with the appropriate

codons for an Iss amino acid sequence or variant thereof. In general, one will select preferred codons for the intended host if the sequence will be used for expression.

The complete sequence is assembled from overlapping nucleotides prepared by standard methods and assembled into a complete coding sequence. See, *e.g.*, Edge,

- 5 Nature, 292:756 (1981); Nambair *et al.*, Science 223:1299 (1984); Jay *et al.*, J. Biol. Chem. 259:6311 (1984). Additionally, synthetic *iss* oligonucleotide sequences may be prepared using an automated oligonucleotide synthesizer as described by Warner DNA, 3:401 (1984).

- 10 Additionally, nucleotide substitutions in SEQ ID NO:22 that can encode a polypeptide having SEQ ID NO:2 or variant thereof can be ascertained by reference to Table 1 and page D1 in Appendix D of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (1989), as well as Table 1 hereinbelow. Nucleotide substitutions can be introduced into DNA segments by methods well known to the art, some of which are described above. See, also, Sambrook *et al.*, Molecular

- 15 Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989).

Likewise, nucleic acid molecules encoding other Iss polypeptides may be modified in a similar manner. Thus, nucleic acid molecules encoding at least a portion of SEQ ID NO:2, or the complement thereto, may be modified so as to yield nucleic acid molecules of the invention having "silent" nucleotide substitutions, or to yield

- 20 nucleic acid molecules having nucleotide substitutions that result in amino acid substitutions (*see* polypeptide or peptide variants hereinbelow).

Table 1

	<u>Amino Acid</u>	<u>Codon</u>
5	Phe	UUU, UUC
	Ser	UCU, UCC, UCA, UCG, AGU, AGC
	Tyr	UAU, UAC
	Cys	UGU, UGC
	Leu	UUA, UUG, CUU, CUC, CUA, CUG
10	Trp	UGG
	Pro	CCU, CCC, CCA, CCG
	His	CAU, CAC
	Arg	CGU, CGC, CGA, CGG, AGA, AGG
	Gln	CAA, CAG
15	Ile	AUU, AUC, AUA
	Thr	ACU, ACC, ACA, ACG
	Asn	AAU, AAC
	Lys	AAA, AAG
	Met	AUG
20	Val	GUU, GUC, GUA, GUG
	Ala	GCU, GCC, GCA, GCG
	Asp	GAU, GAC
	Gly	GGU, GGC, GGA, GGG
	Glu	GAA, GAG

#### **D. Preparation of Expression Cassettes and Vectors and Their Introduction**

##### **25 into Host Cells**

To prepare expression cassettes and vectors for transformation herein, the recombinant or preselected nucleic acid sequence or segment may be circular or linear, double-stranded or single-stranded. As used herein, a "vector," "expression vector," or "expression cassette" is a replicon, or a genetic element that functions as

30 an autonomous unit of DNA replication *in vivo* and capable of replication under its



own control, such as a plasmid, a chromosome, a virus, phage or cosmid. Another DNA segment may be attached to the replicon or genetic element so as to bring about the replication of the attached segment.

Expression cassettes or expression vectors for host cells ordinarily include an  
5 origin of replication, a promoter located upstream from the *iss* coding sequence, together with a ribosome binding site, a polyadenylation site, and a transcriptional termination sequence. Those of ordinary skill will appreciate that some of the aforementioned sequences are not required for expression in certain hosts. For example, an expression vector for use with microbes need only contain an origin of  
10 replication recognized by the host, a promoter that will function in the host and a selection gene.

An expression cassette is constructed according to the present invention so that an avian *E. coli iss* coding sequence is located in the cassette with the appropriate regulatory sequences, the positioning and orientation of the coding  
15 sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (*e.g.*, RNA polymerase that binds to the DNA molecule as the control sequences transcribes the coding sequence). As used herein, a DNA "coding sequence" is that portion of a DNA sequence, the transcript of which is translated into a polypeptide *in vivo* when placed  
20 under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino terminus) and a translation stop codon at the 3' (carboxy terminus). A coding sequence can include, but is not limited to, prokaryotic sequences, genomic DNA sequences from eukaryotic DNA, cDNA from eukaryotic mRNA and even synthetic DNA sequences. A  
25 polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

The control sequences can be ligated to the coding sequence prior to insertion into a cassette. As used herein, a coding sequence is "under the control" of the promoter sequence in a cell when RNA polymerase which binds the promoter

sequence transcribes the coding sequence into mRNA which is then in turn translated into the protein encoded by the coding sequence. Alternatively, the coding sequence can be cloned directly into an expression cassette that already contains the control sequences and an appropriate restriction site.

5

### **1. Transformations**

Transformation may be by any known method for introducing polynucleotides into a host cell, including, for example, packaging the polynucleotide in a virus and transducing a host cell with the virus, and by direct uptake of the polynucleotide. The terms "transformed" or "transformation" or "stably transformed", as used herein, refer to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome. A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced into the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) to chromosomal DNA making up the genome of the cell. In prokaryotes and yeast, for example, the exogenous DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosomal replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

25       The transformation procedure used depends upon the host to be transformed. Bacterial transformation by direct uptake generally employs treatment with calcium or rubidium chloride (Cohen Proc. Natl. Acad. Sci. USA, 69:2110 (1972)). Yeast transformation by direct uptake may be carried out using the method of Hinnen *et al.* Proc. Natl. Acad. Sci., 75:1929 (1978). Mammalian transformations by direct

uptake may be conducted using the calcium phosphate precipitation method of Graham and Van der Eb (Virology, 52:546 (1978), or the various known modifications thereof. Other methods for the introduction of recombinant polynucleotides into cells, particularly into mammalian cells, that are known in the art include dextran-mediated transfection, calcium phosphate mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the polynucleotides into nuclei.

As used herein, the term "recombinant nucleic acid," refers to a nucleic acid that has been derived or isolated from any appropriate source, that may be subsequently chemically altered *in vitro*, and later introduced into target host cells. An example of recombinant DNA "derived" from a source, would be a DNA sequence that is identified as a useful fragment encoding *iss*, or a fragment or a variant thereof, and which is then chemically synthesized in essentially pure form. An example of such DNA "isolated" from a source would be a useful DNA sequence that is excised or removed from said source by chemical means, for example, by the use of restriction endonucleases. The isolated sequence can then be further manipulated, such as, amplified for use in the invention, by the methodology of genetic engineering.

"Recombinant host cells," "host cells," "cells," "cell lines," cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells that can be, or have been used as recipients for a recombinant vector or other transfer DNA, and include the progeny of the original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental or deliberate mutation.

## **2. Cellular hosts**

Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences. Appropriate control sequences that are compatible with the designated host should be used. The term "control sequences" is defined to mean DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The term "operably linked" is defined to mean that the nucleic acids are placed in a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide or fusion polypeptide if it is expressed as a pre-protein that participates in the secretion of the polypeptide or fusion polypeptide; a promoter or enhancer is "operably linked" to a coding sequence if it affects the transcription of the sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

The control sequences that are suitable for prokaryotic cells, for example, include a promoter and optionally an operator sequence, and a ribosome binding site. A "promoter" or "promoter sequence" is a DNA regulatory region to which RNA polymerase binds and initiates transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the translation start codon of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always,

contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

Eukaryotic cells are known to utilize promoters, polyadenylation signals and enhancers. Expression control sequences for prokaryotes include promoters,  
5 optionally containing operator portions and ribosome binding sites. Among prokaryotic hosts, *E. coli* is most frequently used. A number of prokaryotic expression vectors are known in the art. See, for example, U.S. Pat. NOs. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Pub. NOs. GB 2,121,054; GB  
10 2,008,123; GB 2,007,675; and European Pub. No. 103,395.

Transfer vectors compatible with prokaryotic hosts are commonly derived from pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful  
15 transformants by selection. Preferably, expression vectors obtained commercially from Pharmacia are utilized and include pGEX-1 $\lambda$ T (product No. 27-4805-01), pGEX-2T (product No. 27-4801-01), pGEX-2TK (product No. 27-4587-01), pGEX-4T-1 (product No. 27-4580-01), pGEX-4T-2 (product No. 27-4581-01), pGEX-4T-3 (product No. 27-4583-01), pGEX-3X (product No. 27-4803-01), pGEX-5X-1  
20 (product No. 27-4584-01), pGEX-5X-2 (product No. 27-4585-01), pGEX-5X-3 (product No. 27-4586-01), pGEX-6P-1 (product No. 27-4597-01), pGEX-6P-2 (product No. 27-4598-01) and pGEX-6P-3 (product No. 27-4599-01). Expression of *iss* in these vectors yields an GST-Iss fusion protein that can be employed to prepare antibodies, immunogenic compositions, vaccines and diagnostic kits.  
25 Alternatively, the *Iss* polypeptide can be cleaved from GST with PRESCISSON protease (Pharmacia Biotech. Inc.) or other suitable enzyme, purified, and the *Iss* polypeptide can independently be employed to prepare antibodies, immunogenic compositions, vaccines and diagnostic kits.

Commonly used prokaryotic control sequences include the Beta-lactamase (penicillinase) and lactose promoter systems (Chang *et al.* Nature, 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel *et al.* Nucleic Acids Res., 8:4057 (1980)) and the lambda-derived P<sub>L</sub> promoter and N gene ribosome binding site (Shimatake *et al.* Nature, 292:128 (1981)) and the hybrid tac promoter (De Boer *et al.* Proc. Natl. Acad. Sci. USA, 292:128 (1983)) derived from sequences of the trp and lac UV5 promoters. The foregoing systems are particularly compatible with *E. coli*. If desired, other prokaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used with appropriate control sequences. Although the promoters cited above are commonly used, other microbial promoters known in the art, are also suitable.

For routine vector constructions, ligation mixtures are transformed into *E. coli* strain HB101 or other suitable host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants are then prepared according to the method of Clewell *et al.* Proc. Natl. Acad. Sci. USA, 62:1159 (1969), usually following chloramphenicol amplification (Clewell J. Bacteriol., 110:667 (1972)). The DNA is isolated and analyzed, usually by restriction enzyme analysis and/or sequencing. Sequencing may be by the dideoxy method of Sanger *et al.* Proc. Natl. Acad. Sci. USA, 74:5463 (1977) as further described by Messing *et al.* Nucleic Acids Res., 9:309 (1981), or by the method of Maxam *et al.* Methods in Enzymology, 65:499 (1980). Problems with band compression, which are sometimes observed in GC rich regions, are overcome by use of 7-deazoguanosine according to Barr *et al.* Biotechniques, 4:428 (1986).

Eukaryotic hosts include yeast and mammalian cells in culture systems. *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* are the most commonly used yeast hosts and are convenient fungal hosts. Yeast compatible vectors carry markers which permit selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2 micron origin of replication

(Broach *et al.* Meth. Enz., 101:307 (1983)), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences which will result in incorporation of an appropriate fragment into the host cell genome.

Control sequences for yeast vectors are known in the art and include

- 5 promoters for the synthesis of glycolytic enzymes (Hess *et al.* J. Adv. Enzyme Reg., 7:149 (1968)); (Holland *et al.* Biochemistry, 17:4900 (1978)), including the promoter for 3 phosphoglycerate kinase (Hitzeman *et al.* J. Biol. Chem., 255:2073 (1980)). Terminators may also be included, such as those derived from the enolase gene (Holland *et al.* J. Biol. Chem., 256:1385 (1981)). Particularly useful control
- 10 systems are those that comprise the glyceraldehyde-3 phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from GAPDH, and if secretion is desired, leader sequence from yeast alpha factor.

- In addition, an operably linked transcriptional regulatory region and
- 15 transcriptional initiation region do not have to be ones that are naturally associated in a wild-type organism. These systems are described in detail in EPO 120,551, granted August 1, 1990; EPO 116,201, granted April 22, 1992; and EPO 164,556, granted March 2, 1992. Other yeast promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter regions for
- 20 alcohol dehydrogenase 1 or 2, isocytochrome C, acid phosphatase, as well as enzymes responsible for maltose and galactose utilization.

- Mammalian cell lines available as hosts for expression are known in the art. Suitable host cells for expressing Iss in higher eukaryotes include the following:
- 25 monkey kidney CVI line transformed by SV40 (COS-7, ATCC CRL 1651); baby hamster kidney cells (BHK, ATCC CRL 1651); Chinese hamster ovary-cells-DHFR (described by Urlaub and Chasin, PNAS, 77:4216 (1980, USA)); mouse sertoli cells (TM4. Mather, J. P., Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CVI ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells

(MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2 HB 8065); mouse mammary tumor (MMT 060652, ATCC CCL 51); rat hepatoma cells (HTC. M1. 54. Baumann *et al.*, J. Cell Biol., 85:1-8 (1980) and TRI cells (Mather *et al.*,

5 Annals N.Y. Acad. Sci., 383:44-68 (1982)).

Suitable promoters for mammalian cells are also known in the art and include viral promoters such as that from Simian Virus 40 (SV40) (Fiers *et al.* Nature, 273:113 (1978)), Rous sarcoma virus (RSV), adenovirus (ADV), and bovine papilloma virus (BPV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences that increase expression can also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. It will be appreciated that when expressed in mammalian tissue, a recombinant Iss may have higher molecular weight due to glycosylation. It is therefore intended that partially or completely glycosylated forms of Iss having molecular weights greater than provided by the amino acid back-bone are within the scope of this invention.

Vaccinia virus may be used to express foreign DNA and may be used in vaccine preparation. In this case the heterologous DNA is inserted into the Vaccinia genome. Techniques for the insertion of foreign DNA into the vaccinia virus genome are known in the art, and use, for example, homologous recombination. The insertion of the heterologous DNA is generally into a gene that is non-essential in nature, for example, the thymidine kinase gene (tk), which also provides a selectable marker. Plasmid vectors that greatly facilitate the construction of recombinant viruses have been described (see, for example, Mackett *et al.* J. Virol., 49:857 (1984), Chakrabarti *et al.* Mol. Cell Biol. 5:3403 (1985); Moss, Gene Transfer Vectors for Mammalian Cells, (Miller and Calos, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), p.10 (1987)). Expression of the Iss polypeptide then occurs in cells or animal subjects, *e.g.*, birds, cattle, or mink, that are immunized with the live recombinant vaccinia virus.



Other systems for expression of eukaryotic or viral genomes include insect cells and vectors suitable for use in these cells. These systems are known in the art, and include, for example, insect expression transfer vectors derived from the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV), which is a helper-independent, viral expression vector. Expression vectors derived from this system usually use the strong viral polyhedrin gene promoter to drive expression of heterologous genes. Currently the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. The vector pAc373 also contains the polyhedrin polyadenylation signal and the ampicillin-resistance (amp) gene and origin of replication for selection and propagation in *E. coli*. Many other vectors, known to those of skill in the art, have also been designed for improved expression. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 base pairs downstream from the ATT; See Luckow and Summers *Virology*, 17:31 (1989)). Good expression of nonfused foreign proteins usually requires foreign genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal.

Methods for the introduction of heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summer and Smith, Texas Agricultural Experiment Station Bulletin No. 1555; Smith *et al.* *Mol. & Cell Biol.*, 3:2156-2165 (1983); and Luckow and Summers, *supra* (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous recombination. Insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. The inserted sequences may be those which encode all or varying segments of the polypeptide, or other open reading frames ("ORFs") which encode viral polypeptides.

The signals for posttranslational modifications, such as signal peptide cleavage, proteolytic cleavage and phosphorylation, appear to be recognized by insect cells. The signals required for secretion and nuclear accumulation also appear

to be conserved between the invertebrate cells and vertebrate cells. Examples of the signal sequences from vertebrate cells which are effective in invertebrate cells are known in the art, for example, the human interleukin 2 signal (IL2<sub>s</sub>) which is a signal for transport out of the cell, is recognized and properly removed in insect cells.

In a preferred embodiment, the product upon expression of an avian *E. coli* Iss polypeptide yields an Iss-fusion protein that can be purified by affinity chromatography using a commercially available kit with an affinity for the fusion protein (Pharmacia, GST Gene Fusion System, Third Edition, Revision 1 (1997) (product No.18-1123-20), Bulk GST Purification Module (product No. 27-4570-01), Redipack GST Purification Module (product No. 27-4570-02). Once the Iss-fusion protein has been purified, Iss is cleaved from the fusion protein and utilized to immunize mice, *e.g.*, Balb/c. However, an Iss fusion protein can be substituted as the immunogen if desired, although use of Iss alone is preferred. Spleen cells from the immunized mice showing a response to Iss or an Iss fusion protein are fused with myeloma cells to create hybridomas, and hybridomas producing antibody specific to Iss are propagated, characterized and retained for use in immunologic assays to define the association of Iss with avian *E. coli* virulence.

## **E. Polypeptides, Peptides and Variants Thereof**

A recombinant or derived Iss polypeptide, as described above, is not necessarily translated from a designated nucleic acid sequence, for example, SEQ ID NO:22. Iss polypeptides, antigenic variants and subunits thereof, or other Iss subunit polypeptides can be synthesized by the solid phase peptide synthesis (or Merrifield) method or be prepared by a variety of methods known in the art. These and other methods of peptide synthesis are also exemplified by U.S. Pat. NOs. 3,862,925; 3,842,067; 3,972,859; 4,105,602 and 4,757,048.

The Merrifield method is an established and widely used method. It is described in the following references: Stewart *et al.*, Solid Phase Peptide Synthesis,

W. H. Freeman Co., San Francisco (1969); Merrifield, J. Am. Chem. Soc., **85**:2149 (1963); Meinenhofer in Hormonal Proteins and Peptides, Vol. 2, C. H. Li, ed., (Academic Press, 1973), pp. 48-267; and Barany and Merrifield in "The Peptides," Vol. 2, E. Gross and F. Meinenhofer, eds., Academic Press (1980), pp. 3-285.

5           The Merrifield synthesis method commences from the carboxy-terminal end of the peptide using an alpha-amino protected amino acid. Fluorenylmethyloxycarbonyl (Fmoc) or t-butyloxycarbonyl (Boc) protective groups can be used for all amino groups even though other protective groups are suitable, and the first protected amino acids can be esterified to chloromethylated polystyrene resin  
10 supports. The polystyrene resin support is preferably a copolymer of styrene with about 0.5 to 2% divinyl benzene as a cross-linking agent which causes the polystyrene polymer to be insoluble in certain organic solvents. See Carpino *et al.*, J. Org. Chem., **37**:3404 (1972); Meinenhofer, Int. J. Peat. Pro. Res., **11**:246 (1978); and Merrifield, J. Am. Chem. Soc., **85**:2149 (1963). The immobilized peptide is  
15 then N-protected and other amino acids having protected amino groups are added in a stepwise manner to the immobilized peptide. At the end of the procedure, the final peptide is cleaved from the resin, and any remaining protecting groups are removed by treatment under acidic conditions, for example, with a mixture of hydrobromic acid and trifluoroacetic acid. Alternatively, the cleavage from the resin  
20 may be effected under basic conditions, for example, with triethylamine, where the protecting groups are then removed under acidic conditions. The cleaved peptide is isolated and purified by means well known in the art, for example, by lyophilization followed by either exclusion or partition chromatography on polysaccharide gel media such as Sephadex G-25, or countercurrent distribution. The composition of  
25 the final polypeptide may be confirmed by amino acid analysis after degradation of the polypeptide by standard means.

The synthesis may use manual techniques or be completely automated. For example, an Applied BioSystems 431A Peptide Synthesizer (Foster City, Calif.) or a Biosearch SAM II automatic peptide synthesizer (Biosearch, Inc., San Rafael, Calif.)

can be employed following the directions provided in the instruction manual and reagents supplied by the manufacturer. Disulfide bonds between Cys residues can be introduced by mild oxidation of the linear peptide by KCN as taught in U.S. Pat. No. 4,757,048 at column 20.

5           Salts of carboxyl groups of a polypeptide, peptide or variant of the invention may be prepared in the usual manner by contacting the peptide with one or more equivalents of a desired base, for example, a metallic hydroxide base (*e.g.*, sodium hydroxide), a metal carbonate or bicarbonate base, for example, sodium carbonate or sodium bicarbonate, or an amine base, for example, triethylamine or  
10 triethanolamine. Acid addition salts of the peptide may be prepared by contacting the peptide with one or more equivalents of the desired inorganic or organic acid, for example, hydrochloric acid.

          Esters of carboxyl groups of the polypeptides may be prepared by any of the usual methods known in the art for converting a carboxylic acid or precursor to an  
15 ester. One preferred method for preparing esters of the present polypeptides, when using the Merrifield synthesis technique described above, is to cleave the completed polypeptide from the resin in the presence of the desired alcohol either under basic or acidic conditions, depending upon the resin. Thus, the C-terminal of the peptide when freed from the resin is directly esterified without isolation of the free acid.

20           Amides of the polypeptides of the present invention may also be prepared by techniques well known in the art for converting a carboxylic acid group or precursor, to an amide. A preferred method for amide formation at the C-terminal carboxyl group is to cleave the polypeptide from a solid support with an appropriate amine, or to cleave in the presence of an alcohol, yielding an ester. This cleavage is followed  
25 by aminolysis with the desired amine.

          N-acyl derivatives of an amino group of a polypeptide, peptide or variant of the invention may be prepared by utilizing an N-acyl protected amino acid for the final condensation, or by acylating a protected or unprotected peptide. O-acyl derivatives may be prepared, for example, by acylation of a free hydroxy peptide or

peptide resin. Either acylation may be carried out using standard acylating reagents such as acyl halides, anhydrides, acyl imidazoles, and the like. Both N- and O-acylation may be carried out together, if desired. Formyl-methionine, pyroglutamine and trimethyl-alanine may also be substituted at the N-terminal residue of the

5 polypeptide, peptide or variant. Other amino-terminal modifications include aminooxypentane modifications (see Simmons *et al.*, Science 276:276 (1997)).

In addition, the amino acid sequence of an Iss polypeptide can be modified so as to result in a specific polypeptide variant. The modification includes the substitution of at least one amino acid residue in the peptide for another amino acid

10 residue, and includes substitutions that utilize the D rather than L form, as well as other well known amino acid analogs.

One or more residues of the polypeptide or peptide can be altered, so long as the resultant variant is biologically active. Conservative amino acid substitutions are preferred. For example, aspartic-glutamic as acidic amino acids;

15 lysine/arginine/histidine as basic amino acids; leucine/isoleucine, methionine/valine, alanine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids.

Conservative substitutions within the scope of the invention include those shown in Table 2 under the heading of exemplary substitutions. More preferred

20 substitutions are under the heading of preferred substitutions. After the substitutions are introduced, the resulting variants are screened for biological activity.

Table 2

	Original Residue	Exemplary Substitutions	Preferred Substitutions
5	Ala (A)	val; leu; ile	val
	Arg (R)	lys; gln; asn	lys
	Asn (N)	gln; his; lys; arg	gln
	Asp (D)	glu	glu
	Cys (C)	ser	ser
10	Gln (Q)	asn	asn
	Glu (E)	asp	asp
	Gly (G)	pro	pro
	His (H)	asn; gln; lys; arg	arg
	Ile (I)	leu; val; met; ala; phe norleucine	leu
15	Leu (L)	norleucine; ile; val; met; ala; phe	ile
	Lys (K)	arg; gln; asn	arg
	Met (M)	leu; phe; ile	leu
	Phe (F)	leu; val; ile; ala	leu
	Pro (P)	gly	gly
20	Ser (S)	thr	thr
	Thr (T)	ser	ser
	Trp (W)	tyr	tyr
	Tyr (Y)	trp; phe; thr; ser	phe
	Val (V)	ile; leu; met; phe; ala; norleucine	leu
25			

Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gln, his, lys, arg;
- 5 (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic; trp, tyr, phe.

The invention also envisions polypeptide or peptide variants with non-conservative substitutions. Non-conservative substitutions entail exchanging a member of one of the classes described above for another.

- 10 Acid addition salts of the polypeptide, peptide or variant thereof or of amino residues of the polypeptide, peptide or variant may be prepared by contacting the polypeptide, peptide, variant or amine thereof with one or more equivalents of the desired inorganic or organic acid, such as, for example, hydrochloric acid. Esters of carboxyl groups of the polypeptides or peptides may also be prepared by any of the
- 15 usual methods known in the art.

#### **F. Preparation of Iss Antibodies**

- The immunogenic Iss polypeptides or variants thereof prepared as described above are used to produce antibodies, including polyclonal and monoclonal. As
- 20 used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one antibody combining site. An "antibody combining site" or "binding domain" is formed from the folding of variable domains of an antibody molecule(s) to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an
- 25 epitope of an antigen, which allows an immunological reaction with the antigen. An antibody combining site may be formed from a heavy and/or a light chain domain (VH and VL, respectively), which form hyper-variable loops which contribute to antigen binding. The term antibody also includes, for example, vertebrate antibodies, hybrid antibodies, chimeric antibodies, altered antibodies, univalent

antibodies, monoclonal and polyclonal antibodies, the Fab proteins and single domain antibodies.

- If polyclonal antibodies are desired, a selected animal (*e.g.*, mouse, rabbit, goat, horse or bird) is immunized with an immunogenic Iss polypeptide or Iss-fusion protein. Serum from the immunized animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an Iss antigen contains antibodies to other antigens, the polyclonal antibodies can be purified by immuno-affinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker eds.
- 10 Immunochemical Methods in Cell and Molecular Biology (Academic Press, London) (1987).

- Monoclonal antibodies directed against Iss antigens or polypeptides can be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, *e.g.*, Schreier *et al.* Hybridoma Techniques, (1980); Hammerling *et al.* Monoclonal Antibodies and T-cell Hybridomas, (1981); Kennett *et al.* Monoclonal Antibodies (1980); see also, U. S. Pat. NOS. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against Iss antigens can be screened for various properties, for example, isotype, epitope affinity, etc.
- 15  
20

- Preferably, an Iss polypeptide, peptide, variant or subunit thereof, or Iss-fusion protein, is utilized to immunize mice for the preparation of monoclonal antibodies. If it is determined that an Iss polypeptide, a fragment or variant thereof does not elicit a strong humoral response in the immunized mice, a larger and possibly more immunogenic fusion protein (*e.g.*, GST-Iss) can be used as an immunogen. However, regardless of the immunogen used, antibody specificity is determined using Iss alone. Briefly, antigen (an Iss polypeptide, variant or a
- 25



fragment thereof, or GST-Iss) is emulsified in Complete Freund's Adjuvant and the emulsion used to immunize Balb/c mice (about 50-100 µg antigen per mouse given IP). Mice are boosted with an emulsion of antigen-Incomplete Freund's Adjuvant twice at about 10 day intervals (about 50-100 µg antigen each, given IP). About ten  
5 days after the second booster, an antigen-capture ELISA is run to determine the response of the mice to Iss.

The detection of antibody responses specific for the polypeptide can be used in ELISA-based immunoassays for the serodiagnosis of a septicemic disease or virulent, complement resistance *E. coli*. The ELISA is performed by using Iss to  
10 coat wells of microtiter plates. After overnight incubation, coated plates are washed thoroughly, and nonspecific binding sites are blocked. After incubation, plates are thoroughly washed. The primary antibody, *i.e.* antibody contained in the sera from mice immunized with Iss or GST-Iss, is diluted and added to the microtiter plate wells. Following additional washes, a goat anti-mouse IgG- and IgM- alkaline  
15 phosphatase conjugate is added to the wells. After incubation and thorough washing, the substrate for the phosphatase, p-nitrophenyl phosphate, is added to the wells. Plates are incubated in the dark for about 10-45 minutes. Subsequently, changes in absorbance of the plate's contents are read at 405 nm with a microplate spectrophotometer as an indication of mouse response to Iss antigen. With the  
20 identification of a positive antibody, production of monoclonals can proceed. If a positive antibody is not identified, more boosters may be used, or techniques to increase the immunogenicity of Iss can be implemented as stated above.

Alternatively, GST-Iss can be used as the immunogen.

Responding mice are given a final booster consisting of about 5-100 µg,  
25 preferably 25-50 µg of antigen, preferably without adjuvant, administered intravenously. Three to five days after final boosting, spleens and sera are harvested from all responding mice, and sera is retained for use in later screening procedures. Spleen cells are harvested by perfusion of the spleen with a syringe. Spleen cells are collected, washed, counted and the viability determined via a viability assay. Spleen

and SP2/0 myeloma cells (ATCC, Rockville, MD) that have been screened for HAT sensitivity and absence of bacterial contamination are combined, the suspension pelleted by centrifugation, and the cells fused using polyethylene glycol solution. The "fused" cells are resuspended in HT medium (RPMI supplemented with 20 % fetal bovine serum (FBS), 100 units of penicillin per ml, 0.1 mg of streptomycin per ml, 100  $\mu$ M hypoxanthine, 16  $\mu$ M thymidine, 50  $\mu$ M 2-mercaptoethanol and 30 % myeloma-conditioned medium) and distributed into the wells of microtiter plates. Following overnight incubation at 37°C in 5% CO<sub>2</sub>, HAT selection medium (HT plus 4  $\mu$ M aminopterin) is added to each well and the cells fed according to accepted procedures known in the art. In approximately 10 days, medium from wells containing visible cell growth are screened for specific antibody production by ELISA. Only wells containing hybridomas making antibody with specificity to Iss, or GST-Iss, are retained. The ELISA is performed as described above, except that the primary antibody added is contained in the hybridoma supernatants. Appropriate controls are included in each step.

This process generates several hybridomas producing monoclonal antibodies to Iss polypeptides, or an Iss-fusion protein, *e.g.*, GST-Iss. Hybridoma cells from wells testing positive for anti-Iss, or anti-GST-Iss antibodies are cloned by limiting dilution and re-screened for anti-Iss antibody production using ELISA. Cells from positive wells are subcloned to ensure their monoclonal nature. The most reactive lines are then expanded in cell culture and samples are frozen in 90% FBS-10% dimethylsulfoxide. All monoclonal antibodies are characterized using a commercial isotyping kit (BioRad Isotyping Panel, Oakland, CA) and partially purified with ammonium sulfate precipitation followed by dialysis. Further purification is performed using protein-A affinity chromatography.

Antibodies, both monoclonal and polyclonal, that are directed against Iss antigens are particularly useful in diagnosis. Those that are neutralizing are useful in passive immunotherapy and treatment. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies.

The term "treatment," as used herein, refers to prophylaxis and/or therapy of a subject diagnosed with, at risk of, or exhibiting characteristics or symptoms of, various *E. coli* infections including, but not limited to, septicemic disease, colibacillosis, coligranuloma, peritonitis, salpingitis, synovitis, and omphalitis. The term "therapy" refers to providing therapeutic benefit or effect to a subject afflicted with the disease such that the subject exhibits few or no symptoms of a septicemic disease or other related diseases. The term "prophylaxis" refers to administration of treatment prior to a subject who is or may become at risk for contracting the disease. Such treatment can be accomplished by administration of nucleic acids, polypeptides or antibodies of the invention. When the subject is a cow, treatment can also be used to prophylactically treat a calf *in utero* or after birth by immunizing a cow before, during, or after pregnancy. For example, it is expected that maternal anti-Iss antibodies will be passively transferred from the dam to the offspring via colostrum. Optionally, colostrum can be obtained from a cow that has been immunized using the methods herein, and fed to a calf diagnosed with, at risk of, or exhibiting characteristics or symptoms of various *E. coli* infections including scours and septicemic disease.

#### **G. Preparation of Immunogenic Compositions and Vaccines of the Invention**

The preparation of immunogenic compositions or vaccines that contain immunogenic polypeptide(s), peptides, and polypeptides encoded by the nucleic acid molecules of the invention as an active ingredient are known to one skilled in the art. As used herein, the term "immunogenic composition" refers to a composition or preparation administered in an amount effective to raise antibodies in a recipient and further provides some therapeutic benefit or effect so as to result in an immune response that inhibits or prevents a septicemic disease in a subject, or so as to result in the production of antibodies to a virulent complement resistant avian *E. coli* isolate, or polypeptide or peptide employed as an immunogen. Both local and systemic administration is contemplated. Systemic administration is preferred.

The term "vaccine" refers to the process of immunization and the administration of an antigen or a suspension of antigens, derived from either bacteria or viruses, that upon administration, will produce active immunity and provide protection against those viruses or bacteria or related viruses or bacteria, utilizing either conventional, traditional or recombinant techniques, *e.g.* those involving recombinant DNA technology or synthetic peptides. The antigen or suspension of antigens can be encoded by a nucleic acid molecule that is administered to the subject. Typically, such immunogenic compositions and vaccines are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the polypeptide encapsulated in liposomes.

The active immunogenic ingredients are often mixed with excipients or diluents that are pharmaceutically acceptable as carriers and compatible with the active ingredient. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the immunogenic composition or vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the immunogenic composition or vaccine.

Examples of adjuvants or carriers that may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP); N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL + TDM + CWS) in a 2% squalene/Tween 80 emulsion. The effectiveness of an

adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing an Iss antigenic sequence resulting from administration of the polypeptide (or a nucleic acid encoding the polypeptide) in immunogenic compositions or vaccines that are also comprised of the various  
5 adjuvants.

## **H. Dosages, Formulations and Routes of Administration of Nucleic Acid**

### **Molecules and Polypeptides of the Invention**

The nucleic acid molecules, polypeptides or peptides of the invention are  
10 preferably administered to a subject so as to result in an immune response specific for the polypeptide, including the polypeptide encoded by the nucleic acid molecules of the invention or peptide. The immunogenic compositions and vaccines of the present invention are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly either as liquid solutions or  
15 suspensions. Solid forms suitable for suspension in a liquid vehicle prior to injection or infusion can also be prepared. Additional formulations that are suitable for administration include oral formulations. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium  
20 carbonate and the like. Iss polypeptides may be formulated into an immunogenic composition or vaccine as neutral or salt forms. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 10%-95% of active ingredient, preferably about 25%-70%.

25 Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium,

potassium, ammonium, calcium, or ferric hydroxides and such organic bases as isopropylamine, trimethylamine, ethylamino ethanol, histidine, procaine, and the like.

Immunogenic compositions and vaccines comprising nucleic acid molecules, polypeptides or peptides of the instant invention are administered to an animal, *e.g.*, birds, cattle, or mink, in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective and result in an immune response that is specific for an Iss polypeptide, Iss peptide, or Iss polypeptide encoded by a nucleic acid molecule. It is very common to express dosage units in mg/kg (*i.e.*, mg/kg of body weight) or, if a continuing series of doses over many days is contemplated, mg/kg/day. In unit dose form, an immunogenic composition or vaccine of the invention will usually contain an effective amount, *e.g.*, an amount capable of eliciting an immune response in a subject, of an Iss polypeptide in conjunction with a conventional, pharmaceutically acceptable carrier.

The dosage will vary depending upon the specific purpose for which the protein is being administered. The prepared compounds and compositions can be administered to subjects for veterinary use, such as for use with domestic or farm animals.

In general, the dosage of polypeptide required for efficacy will range from about 0.003 to 100 mg/kg, preferably about 0.05 to 50 mg/kg, and more preferably 0.5 to 30 mg/kg, although other dosages can provide beneficial effects. A dosing method as described in Borch *et al.* U.S. Patent No. 5,035,878, provides additional guidance. Although Borch *et al.* is directed to mammals, the weight of a subject can readily be substituted. Dosage, however, may depend on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend on the judgment of the practitioner and may be peculiar to the subject to be immunized.

A nucleic acid molecule used in an immunogenic composition or a vaccine of the invention is preferably one that includes a nucleotide sequence encoding a polypeptide that includes an *E. coli* Iss polypeptide or an immunogenic subunit thereof. The nucleic acid molecule can include DNA, RNA, a modified nucleic acid, or any combination thereof. The nucleic acid molecule can be supplied as part of a vector or as a "naked" nucleic acid molecule. General methods for construction, production and administration of nucleic acid molecule vaccines are known in the art, e.g. F. Vogel et al., *Clin. Microbiol. Rev.* 8:406-410 (1995); WO 93/02556; Felgner et al., U.S. Patent No. 5,580, 859, Pardoll et al., *Immunity* 3:165 (1995); Stevenson et al., *Immunol. Rev.* 145:211 (1995); Molling, *J. Mol. Med.* 75:242 (1997); Donnelly et al., *Ann. N.Y. Acad. Sci.* 772:40 (1995); Yang et al., *Mol. Med. Today* 2:476 (1996); and Abdallah et al., *Biol. Cell* 85:1 (1995)). A nucleic acid molecule can be generated by means standard in the art, such as by recombinant techniques, or by enzymatic or chemical synthesis.

Preferably, administration of a nucleic acid molecule that is part of an immunogenic composition or a vaccine includes the introduction of an expression vector that includes the nucleic acid molecule. There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccine plasmids. A specific embodiment employs constructs using the plasmid pVAX1 as the vector (InVitrogen Corporation, Carlsbad, CA). In addition, the vector construct can contain immunostimulatory sequences that stimulate the animal's immune system. Examples of immunostimulatory sequences include, for instance, sequences with CpG motifs, two 5' purines, an unmethylated CpG dinucleotide, or two 3' pyrimidines (see, for instance, Lowie et al., *DNA Vaccines Methods and Protocols*, Humana Press, Totowa, NJ (2000)). Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences coding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF) or interleukin-12 (IL-12). The cytokines can be used in various combinations to fine-tune the response of the animal's immune system, including both antibody

and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to produce an immune response. Alternatively, the vector can be a viral vector, including an adenovirus vector, and adenovirus associated vector, or a retroviral vector.

- 5 Promoters and terminators useful in a nucleic acid molecule used in an immunogenic composition or vaccine are able to cause, in the appropriate type of cell (*e.g.*, an avian cell), expression of the coding sequence to which the promoter and terminator are operably linked. Examples of promoters include, for instance, a cytomegalovirus promoter (see for instance, U.S. Patent 5,168,062 (Stinski)), a Rous  
10 Sarcoma Virus promoter (for instance promoter from the Rous Sarcoma Virus loing terminal repeat), a chicken  $\beta$ -actin promoter. Preferably, when the subject is a bird, the promoter is a chicken  $\beta$ -actin promoter (Heckert et al., "An enhanced Plasmid Expression Vector for Avian DNA Vaccination," XXI World's Poultry Congress, Montreal, Canada, August 20-24 (2000)). Examples of terminators include, for  
15 instance, the bovine growth hormone terminator sequence (Goodwin et al., *J. Biol. Chem.*, 267, 16330-16334 (1992)), and a synthetic polyadenylation termination sequence (Levitt et al., *Genes Dev.*, 3, 1019-25 (1989)).

- A nucleic acid molecule is preferably administered in one or more doses containing the plasmid, viral vector or naked polynucleotide in an amount of about 5  
20  $\mu$ g to about 100  $\mu$ g. One of skill in the art can readily determine a suitable dosage for a particular animal, depending on the nature, size and overall health of the animal, as well as the condition to be treated. Optionally, the immune response that results from administration of the nucleic acid molecule can be enhanced by methods known to the art including, for instance, encapsulating the nucleic acid  
25 molecule for controlled release, mixing the nucleic acid molecule with cytokines, administering the nucleic acid molecule with co-stimulatory plasmids or other molecular adjuvants, varying the polyadenylation site, or fusing the sequence encoding the *E. coli* Iss polypeptide with a sequence encoding a second polypeptide



(see, e.g., Lowie et al., DNA Vaccines Methods and Protocols, Humana Press, Totowa, NJ, 145-220 (2000)).

An immunogenic subunit of an *E. coli* Iss polypeptide is a subunit that elicits an immune response in a subject to which it is administered. An immune response  
5 includes either or both of a cellular immune response or production of antibodies, and can include activation of the subject's B cells, T cells, helper T cells or other cells of the subject's immune system. For example, an immune response is evidenced by a detectable anti-*E. coli* Iss polypeptide antibody level in the subject using methods of antibody detection known to the art.

10 Immunogenicity of an *E. coli* Iss polypeptide or subunit can be determined, for example, by administering the adjuvanted candidate subunit to the subject, then observing of the associated immune response by analyzing anti-*E. coli* Iss subunit antibody titers in serum. An immunogenic subunit preferably contains more than 7 amino acids, more preferably at least about 10 amino acids, most preferably at least  
15 about 20 amino acids. When an immunogenic subunit of an *E. coli* Iss polypeptide or subunit is identified, the nucleic acid sequence encoding the subunit can be inserted into an appropriate expression vector, for instance pVAX1.

The immunogenic composition or vaccine can be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one  
20 in which a primary course of vaccination may be with approximately 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at about 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the subject  
25 and be dependent upon the judgment of the practitioner. In addition, the immunogenic composition or vaccine containing an immunogenic Iss antigen(s), Iss polypeptide or Iss fusion polypeptide can be administered in conjunction with other immunoregulatory agents, for example, immune globulins.

## **I. Immunoassay and Diagnostic Kits**

Polypeptides that react immunologically with serum containing Iss antibodies or Iss-fusion protein antibodies are useful in immunoassays to detect presence of avian *E. coli* Iss in biological samples. Examples of useful antibodies

- 5 include those derived from, expressed from, or encoded within the Iss clones described in Example 1, and composites thereof, and the antibodies raised against the Iss antigens in these polypeptides.

- Design of an immunoassay is subject to a great deal of variation, and many formats are known in the art. Ideally the immunoassay will utilize at least one  
10 antigen derived from an avian *E. coli* Iss. In one embodiment, the immunoassay can utilize a combination of antigens derived from Iss or an Iss fusion polypeptide. These antigens can be derived from the same or from different Iss polypeptides, and may be in separate recombinant or natural polypeptides, or together in the same recombinant polypeptides.

- 15 An immunoassay may use, for example, a monoclonal antibody directed towards an Iss antigen(s) or Iss fusion polypeptide, a combination of monoclonal antibodies directed towards several Iss antigens or Iss fusion polypeptides, polyclonal antibodies directed towards the same Iss antigen or Iss fusion polypeptide, or polyclonal antibodies directed towards different Iss antigens or Iss  
20 fusion polypeptides. Protocols may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide. The labels may be, for example, enzymatic, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals  
25 from the probe are also known. Examples of these are assays that utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Typically, an immunoassay for an anti-Iss antibody(s) will involve selecting and preparing the test sample suspected of containing the antibodies, such as a biological sample, then incubating it with an antigenic (*i.e.*, epitope-containing) Iss

polypeptide(s) or Iss fusion polypeptide(s) under conditions that allow antigen-antibody complexes to form, and then detecting the formation of such complexes. Suitable incubation conditions are well known in the art. The immunoassay may be, without limitation, in a heterogeneous or in a homogeneous format and of a standard or competitive type.

In a heterogeneous format, the polypeptide is typically bound to a solid support to facilitate separation of the sample from the polypeptide after incubation. Examples of solid supports that can be used are nitrocellulose (*e.g.*, in membrane or microtiter well form), polyvinyl chloride (*e.g.*, in sheets or microtiter wells), polystyrene latex (*e.g.*, in beads or microtiter plates, polyvinylidene fluoride (for instance IMMUNLON)), diazotized paper, nylon membranes, activated beads and Protein A beads. For example, Dynatech IMMUNLON 1 or IMMUNLON 2 microtiter plates or 0.25 inch polystyrene beads (Precision Plastic Ball) can be used in the heterogeneous format. The solid support containing the antigenic polypeptide is typically washed after separating it from the test sample, and prior to detection of bound antibodies. Both standard and competitive formats are known in the art.

In a homogeneous format, the test sample is incubated with antigen in solution. For example, it may be incubated under conditions that will precipitate any antigen-antibody complexes that are formed. Both standard and competitive formats for these assays are known in the art.

In a standard format, the amount of Iss antibodies forming the antibody-antigen complex is directly monitored. This may be accomplished by determining whether labeled anti-xenogenic antibodies that recognize an antigen on anti-Iss antibodies will bind due to complex formation. In a competitive format, the amount of Iss antibodies in the sample is deduced by monitoring the competitive effect on the binding of a known amount of labeled antibody (or other competing ligand) in the complex.

Complexes formed comprising anti-Iss antibody (or, in the case of competitive assays, the amount of competing antibody) are detected by any of a

number of known techniques, depending on the format. For example, unlabeled *Iss* antibodies in the complex may be detected using a conjugate of anti-xenogenic Ig complexed with a label, (e.g., an enzyme label).

In immunoassays where *Iss* polypeptides are the analyte, the test sample, typically a biological sample, is incubated with anti-*Iss* antibodies under conditions that allow the formation of antigen-antibody complexes. Various formats can be employed. For example, a "sandwich assay" may be employed, where antibody bound to a solid support is incubated with the test sample; washed; incubated with a second, labeled antibody to the analyte, and the support is washed again. Analyte is detected by determining if the second antibody is bound to the support. In a competitive format, which can be either heterogeneous or homogeneous, a test sample is usually incubated with antibody and a labeled, competing antigen is also incubated, either sequentially or simultaneously. These and other formats are well known in the art.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials. These kits include the polypeptides of the invention containing *Iss* antigens, or antibodies directed against *Iss* epitopes, in suitable containers, along with the remaining reagents and materials required for the conduct of the assay packaged in preselected amounts, as well as a suitable set of assay directions. All of these items are contained within the outer packaging of the kit, which may be a box, envelope, or the like. The assay directions preferably comprises instruction means such as a printed insert, a label, a tag, a cassette tape and the like, instructing the user in the practice of the assay format.

Additionally, *iss* probes can be packaged into diagnostic kits. Such diagnostic kits can include avian *E. coli iss* nucleic acid probe(s), that may be labeled. As used herein, the term "probe" refers to a polynucleotide that forms a hybrid structure with a sequence in a target region, due to complementarity of at least one sequence in the probe with a sequence in the target region. Specifically a

probe is a nucleic acid sequence between 10 and 500 base pairs in length that contains specific nucleotide sequences that specifically and preferentially hybridize under predetermined conditions to nucleic acid sequences of an avian *E. coli iss* nucleic acid sequence.

5           Alternatively, the probe DNA may be unlabeled and the ingredients for labeling the probe may be included in separate containers in the kit. The kit may also contain other suitably packaged reagents and materials needed for the particular hybridization protocol (*e.g.*, standards) as well as instructions for conducting the test.

10           For example, one such diagnostic kit for detecting or determining antibodies to Iss or an Iss fusion polypeptide comprises packaging containing, separately packaged: (a) a solid surface, such as a fibrous test strip, a multi-well microtiter plate, a test tube, or beads, having bound thereto a polypeptide, peptide, variant, or subunit, of SEQ ID NO:2 and (b) a labeled immunoglobulin that will bind to an  
15   antibody produced by the subject. A second embodiment of a diagnostic kit for detecting or determining Iss comprises packaging containing, separately packaged: (a) a solid surface having bound thereto antibodies to the polypeptide of SEQ ID NO:2 or a fragment thereof; and (b) a known amount of (i) antibodies specific to Iss or an Iss fusion polypeptide or (ii) antibodies to Iss that comprise a detectable label,  
20   or a binding site for a detectable label. A third embodiment of a diagnostic kit for detecting Iss or an Iss fusion polypeptide can comprise, in packaged association, separately packaged amounts of: (a) an Iss-specific antibody; (b) the labeled polypeptide of SEQ ID NO:2 or a fragment thereof, and (c) an immunoglobulin that will bind to an antibody produced by the subject.

25

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

### Example 1

#### Identification, cloning and sequence of an avian *E. coli iss* gene

- A wild-type *E. coli* isolate was obtained from the serum of a chick with systemic colibacillosis. The isolate was identified as an O2 serotype. The virulence of the isolate was determined by embryo lethality assay. Twelve-day-old embryonated eggs were obtained from Seaboard, Athens, Georgia. Overnight cultures of the isolates that were tested, including virulent and avirulent control organisms, were pelleted by centrifugation, washed twice in phosphate-buffered saline (1X PBS) and resuspended to a concentration of  $10^2$  colony-forming units per 0.10 ml of PBS. The final concentration was then confirmed by viable counts. The 0.1-ml inoculum was then injected into the allantoic cavity of each embryo. Twenty embryos per isolate were utilized. Inoculated embryos were incubated at 37°C and counted daily for 4 days to identify dead embryos (Minshew *et al.*, Infect Immun. 20:50-54 (1978); Nolan *et al.*, Avian Dis. 36:395-397 (1992)).
- Inoculation of the isolate into chick embryos resulted in death of 98% of the embryos, indicating that the isolate was virulent. The isolate also was resistant to the lytic effects of complement, which did not degrade C3, and limited C3 deposition on the cell surface of the bacterium when compared with a complement-sensitive mutant of this wild-type isolate.
- The isolate contained several plasmids including a 100-kb plasmid that produced Colicin V. The isolate also produced the siderophores, enterobactin and aerobactin. The isolate was non-hemolytic on blood agar (Nolan *et al.*, Avian Dis., 36:398-402 (1992)), motile (Nolan *et al.*, Avian Dis., 36:395-397 (1992)), and lacked K1 antigen, capsule, and type I pili. Additionally, the isolate contained the *traT* (Nolan *et al.*, Avian Dis., 38:146-150 (1994)) and *iss* genes, had a smooth LPS (Nolan *et al.*, Avian Dis., 38:146-150 (1994)), was resistant to rough-specific bacteriophages (Nolan *et al.*, Avian Dis., 38:146-150 (1994)) and to the antibiotics, streptomycin, sulfisoxazole and tetracycline.

The complete *iss* gene from this isolate was obtained by PCR amplification utilizing the following oligonucleotide primer pair:

5'-GTGGCGAAACTAGTAAAACAGC-3' (SEQ ID NO:3) and

5'-CGCCTCGGGGTGGATAA-3' (SEQ ID NO:4).

- 5 The primer pair was selected using DNASTAR's Primer Select Program (Madison, WI). The resultant 760 bp PCR product (SEQ ID NO:1) was sequenced and cloned into a pGEM-T vector (Promega Corp., Madison, WI) (Sambrook, Fitsch & Maniatis, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989); DNA Cloning, Volumes I and II (D. N. Glover ed. 1985)). The
- 10 resulting *iss* plasmid clones were also sequenced to confirm their identities using standard procedures with a LICOR™ automated sequencer (Lincoln, NE). The *iss* DNA sequence was compared with the *iss* sequence from an isolate of a human *E. coli* and the sequence of lambda *bor* (Barondess *et al.*, J. Bacteriol., 177:1247-1253 (1995); Barondess *et al.*, Nature, 346:871-874 (1990); Chuba *et al.*, Mol. Gen.
- 15 Genet., 216:287-292 (1989)) using Lasergene Software (DNASTAR) and FASTA (EMBL, Heidelberg, Germany). The DNA sequence alignment of those three genes is shown in Figure 1. The avian *E. coli iss* sequence was submitted to GENBANK on January 10, 1998, and has been assigned accession number AF042279.

20

## **Example 2**

### **Expression of *iss***

- Two amplified *iss* sequences, one containing a full-length *iss* sequence as shown in Figure 1 (SEQ ID NO:22), and the other containing a truncated *iss* sequence wherein nucleotides 1-72 (Figure 1) were deleted, were cloned in frame
- 25 into the expression vector, pGEX-6P-3 (Pharmacia Biotech Inc., Piscataway, N.J.), generating plasmid clones pLN321 and pLN322 respectively. The pGEX-6P-3 vector was used in combination with a GST Gene Fusion System Purification Kit (Pharmacia Biotech Inc., Piscataway, NJ) and provided a complete system for expressing and purifying resulting Iss polypeptides.

Once cloned into the pGEX-6P-3 expression vector, both plasmid clones were sequenced and shown, by DNA sequencing, to be fused to the GST vector in the proper reading frame (Figure 3, showing pLN321 only). The pLN321 construct was then used to transform a protease-deficient *E. coli* strain BL21 (Pharmacia Biotech Inc., Piscataway, N.J.). Protein expression was induced with IPTG (Pharmacia Biotech Inc., Piscataway, N.J.). Following induction, the *E. coli* were lysed and the lysates were analyzed by SDS-PAGE. The crude total protein preparations showed that the uninduced and induced *E. coli* containing these constructs differed in their expression of a 37-kD protein band (Figure 4). The 37-kD band, lane 3I of Figure 4, corresponds in size to the predicted Glutathione S-Transferase-Iss (GST-Iss) fusion protein, as the Iss polypeptide has a molecular weight of approximately 10-11 kD, and GST has a molecular weight of approximately 26 kD. The 37-kD product obtained from the *iss*-pGEX-6P-3 construct, was found in the induced bacterial lysate, and not in the uninduced lysate.

To further confirm the identity of the 37-kD band observed in the induced bacterial lysate, the proteins of these lysates were transferred from the SDS-PAGE gel to a PVDF membrane (BioRad, Oakland, CA). The PVDF membrane was then probed with a GST monoclonal antibody (Pharmacia Biotech Inc., Piscataway, N.J.)). After washing, bound antibody was detected using a secondary antibody conjugated to alkaline phosphatase in the presence of the color reagent, BCIP/NBT enzyme substrate (BioRad, Oakland, CA). The 37-kD band was recognized by anti-GST, thereby confirming that the 37-kD band represented a GST-Iss fusion product (Figure 5).

### **Example 3**

#### **Purification and isolation of Iss**

Polypeptides prepared from the pGEX-6P-3 expression vector yielded a glutathione S-transferase-Iss ("GST-Iss") fusion polypeptide product that is readily purified from the bacterial lysates by affinity chromatography under mild, non-



denaturing conditions. Specifically, a bacterial sonicate is applied to a column of glutathione sepharose 4B at 4° C and washed three times with 10 bed volumes of 1X PBS. Glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0)) is added to the column and incubated at room temperature (about 22-  
5 25° C) for 10 minutes, and the fusion protein is eluted. Eluates recovered from the column contain the fusion protein.

Alternatively, after expression of GST-Iss in *E. coli*, the bacteria are lysed by sonication, and the insoluble material is pelleted and removed, and the supernatant passed through a slurry of Glutathione Sepharose 4B (Pharmacia Biotech Inc.,  
10 Piscataway, N.J.) to permit binding of the GST-Iss fusion polypeptide to the Sepharose beads. To remove GST-Iss from other cellular proteins, the "bead-bound" fusion polypeptide is pelleted by centrifugation and washed with 1X PBS. The desired product is eluted from the Sepharose by the addition of reduced glutathione (Pharmacia Biotech Inc., Piscataway, NJ). Upon removal from the  
15 Sepharose beads, the purified GST-Iss fusion product is cleaved into Iss and GST by a site-specific protease, such as PreScission Protease (Pharmacia Biotech Inc., Piscataway, NJ), and the remaining GST is separated from Iss by the same procedure used to purify the GST-Iss fusion polypeptide. The resulting polypeptide products are then analyzed by SDS-PAGE.

20

#### **Example 4**

##### **Presence of iss in avian *E. coli* isolates**

Two hundred and ten *E. coli* isolates from poultry clinically diagnosed with colibacillosis were obtained from several locations in the United States. Fifty-six *E.*  
25 *coli* isolates from the feces of apparently healthy poultry were obtained from the University of Georgia or were from poultry in North Dakota. The identity of all *E. coli* isolates was confirmed using API20E Strips (bioMerieux, Vitek, Inc., Hazelwood, MO). Non-*E. coli* strains (Table 3) used to evaluate the specificity of the probes were obtained from the NDSU Veterinary Diagnostic Laboratory.

Table 3

	<i>Bacillus subtilis</i>	<i>Micrococcus luteus</i>
	<i>Citrobacter freundii</i>	<i>Proteus mirabilis</i>
5	<i>Enterobacter aerogenes</i>	<i>Pseudomonas fluorescens</i>
	<i>Enterobacter cloacae</i>	<i>Rhodococcus erythropolis</i>
	<i>Hafnia alvei</i>	<i>Staphylococcus aureus</i>
	<i>Klebsiella oxytoca</i>	<i>Staphylococcus epidermidis</i>
	<i>Klebsiella pneumoniae</i>	<i>Streptococcus faecalis</i>
10	<i>Salmonella typhimurium</i> (Copenhagen)	

All isolates were maintained in LB broth (Difco Laboratories, Detroit, Mich.) supplemented with 20% glycerol and stored at -70° C prior to use. When subjected to total DNA isolation procedures, sample isolates were grown in BHI (Difco Laboratories, Detroit, Mich.), with or without antibiotics, overnight at 37° C. In preparation for amplification procedures, isolates were grown on MacConkey agar (Difco Laboratories, Detroit, Mich.) overnight at 37° C. For colony blotting procedures, test organisms were grown on LB agar (Difco Laboratories, Detroit, Mich.) overnight at 37° C.

#### Probe construction

A *cvaC* probe was prepared by digesting plasmid pHK11 (Dr. R.E. Wooley, University of Georgia, Athens, GA) with the restriction enzymes *EcoRI* and *BglII*, which were obtained from Promega Corp. (Madison, WI.) to yield a 1.9 kb fragment (Gilson *et al.*, J. Bacteriol. 169:1466-2470 (1987)). To obtain a *traT* probe (Moll *et al.*, Infect. Immun., 28:359-367 (1980); Montenegro *et al.*, J. Gen. Microbiol., 131:1511-1521 (1985)), the plasmid pKT107 (Dr. F.C. Cabello, New York Medical College, Valhalla, NY) was digested with *BstEII* to yield a 700 bp fragment.

Gene probes for *ompA* and *iss* were generated through DNA amplification techniques. Primer sequences useful to amplify *ompA* and *iss* sequences were selected using Lasergene software (DNASTar, Inc., Madison, WI.) based on published sequences for these genes (Beck *et al.*, Nuc. Acids Res., 8:3011-3024

(1980); Chuba *et al.*, Mol. Gen. Genet., 216:287-292 (1989)) and were obtained from Genosys Biotechnologies, Inc., The Woodlands, Tex. Primers employed in the detection of *ompA* and *iss* are shown in Table 4.

5

Table 4

Name	Sequences (5' to 3')	SEQ ID NO.	Tm
<i>ompA</i>			
upper primer	CTTGCGGAGGCTTGTCTGAG	9	54.9°C
lower primer	AGGCATTGCTGGGTAAGGAA	10	53.7°C
<i>iss</i>			
upper primer	GTGGCGAAAAC TAGTAAACAGC	3	52.1°C
lower primer	CGCCTCGGGGTGGATAA	4	53.9°C
<i>iss</i>			
issupper1ex	AAAGGGGATCCATGCAGGATAATA AGATGAAAAA	11	73.6°C
issupper73ex	CACAGGGATCCCAAACGTTTACTG TTGGAAACAA	12	77.8°C
isslower338ex	CGCCGGAATTTCGCAGATGAGCTCC CCATATC	13	82.4°C
issupperdiag	ATGCAGGATAATAAGATGAAAAA	14	47.6°C
isslowerdiag	ATAGATGCCAAAAGTGATAAAAC	15	47.2°C

30

*ompA* and *iss* sequences were amplified according to the following procedure. A single colony of an *E. coli* isolate was transferred into 20 µl of Gene Releaser™ (Bioventures, Inc., Murfreesboro, Tenn.) and subjected to microwaves on the high setting for approximately 6 minutes (Kenmore, ultra-defrost). A PCR master mix consisting of 47.5 µl of H<sub>2</sub>O, 10.0 µl of 10X PCR Buffer II (Perkin

Elmer, Branchburg, NJ), 16.0  $\mu$ l of 1.25 mM dNTP mix (Promega), 0.5  $\mu$ l of Taq DNA Polymerase (Promega, Madison, WI), 1.0  $\mu$ l of 0.1 mM of each primer, and 4.0  $\mu$ l of 25 mM  $MgCl_2$  was added to an *E. coli* - Gene Releaser<sup>TM</sup> suspension. Amplification was performed according to the following parameters: 2 minutes at 97° C; 1 minute at 97° C; 1 minute at 49° C, 1 minute at 72° C for 9 cycles; 1 minute at 95° C, 1 minute at 49° C, 1 minute at 72° C for 24 cycles; 5 minutes at 72° C and then the sample is maintained at 4° C.

Restriction enzyme digest plasmid fragments and amplification products were separated by horizontal gel electrophoresis. Amplified fragments were identified by size, excised from the agarose and purified using GENE CLEAN<sup>TM</sup> (Bio101, La Jolla, Calif.) or the Wizard PCR Clean-Up System (Promega, Madison, WI). The identities of the amplicons were further confirmed by sequencing according to the procedures described below. To prepare probes, isolated fragments were labeled using a non-radioactive, random-primed DNA labeling kit (Genius I Labeling and Detection Kit, Boehringer Mannheim, Indianapolis, Ind.).

#### Sequencing of amplified DNA

Sequencing was performed according to the manufacturer's protocol using the LI-COR 4000LR Automated DNA Sequencer (Lincoln, Neb.). Briefly, 9  $\mu$ l of each purified PCR product, containing 0.1 to 0.6 pmol DNA, was added to 2  $\mu$ l of 1 pmol/ $\mu$ l of the appropriate primer DNA (for example, SEQ ID NOs: 3, 4, 9 and 10 in Table 4) 1.0  $\mu$ l of dNTP mix, 1.0  $\mu$ l of IRD40-dATP at 20 pmol/ $\mu$ l (Boehringer Mannheim, Indianapolis, Ind.), 2.5  $\mu$ l of 10x Sequitherm<sup>TM</sup> Reaction Buffer (Epicentre Technologies, Madison, WI.), and 1.5  $\mu$ l of Sequitherm<sup>TM</sup> DNA Polymerase at 5 u/ $\mu$ l (Epicentre, Madison, WI). All components were mixed carefully in labeled tubes and covered with mineral oil. The tubes containing this mixture were placed in the thermal controller, and the cycle sequencing reaction was run as described in LI-COR (Lincoln, Neb.) application bulletin 41.

The first series of cycles were the labeling reactions, wherein an IRD40-dATP was added to the extended primer sequence. The second series of cycles was the termination reaction for which 4 µl of the labeling reaction was added to 2 µl of each ddNTP termination mix before cycling. Unincorporated label was removed from the products by ethanol precipitation according to manufacturer's directions. Samples were separated on 4.0 % Long Ranger acrylamide gels (FMC, Rockland, ME) and analyzed via a LI-COR 4000 LR automated sequencer.

#### Amplification for cloning

*ompA* and *iss* sequences used for cloning were amplified according to the following procedure. A single colony of *E. coli* isolate was transferred into 20 µl of Gene Releaser™ (Bioventures, Inc., Murfreesboro, Tenn.) and subjected to microwaves on the high setting for approximately 6 minutes (Kenmore, ultra-defrost). Alternatively, a colony was transferred into 40 µl SCLB (10 mM Tris-HCl pH 7.5/ 1 mM EDTA/ 50 µg/ml proteinase K), and heated at 55 °C for 10 minutes, then heated at 80 °C for 10 minutes, diluted with 80 µl ddH<sub>2</sub>O, cell debris was pelleted and 10 µl of the supernatant was used.

The prepared *E. coli* DNA was added to a master mix consisting of 47.5 - 53.5 µl ddH<sub>2</sub>O, 10.0 µl of 10X PCR Buffer II (Perkin Elmer or Promega, Madison, WI.), 0.5 µl of Amplitaq DNA polymerase (Perkin Elmer), 1.0 µl of 0.1 mM of each appropriate primer and 4.0 µl or 8.0 µl of 25 mM MgCl<sub>2</sub>.

The amplification cycles were as previously described except that the annealing temperature was 49 °C or 51.8 °C depending on the primer pair used. Amplified fragments for ligation into the expression vector pGEX-6P-3, were digested with *Bam*HI and *Eco*RI to produce sticky ends for the ligation process. Amplified fragments for ligation into pGEM-T vector have "A" overhangs left by Taq polymerase. Restriction enzyme digest plasmid fragments and amplification products were separated by horizontal gel electrophoresis. Amplified fragments were identified by size, excised from the agarose and purified using the Wizard PCR

Clean-Up System (Promega, Madison, WI). T7 DNA ligase was used to ligate amplification fragments into the vectors. The identities of the amplicons were further confirmed by sequencing according to the procedures described below.

## 5 Sequencing of cloned DNA

Sequencing was performed according to the manufacturer's protocol using the LI-COR 4000LR Automated DNA Sequencer (Lincoln, Neb.). Briefly, 9 µl of each purified plasmid clone containing 0.2 to 0.6 pmol DNA, was added to 1 µl of 1 pmol/µl IRD41 labeled primer DNA (Table 5), 2.5 µl of 10x Sequitherm™ Reaction Buffer (Epicentre Technologies, Madison, WI.), and 1.0 µl of Sequitherm™ DNA Polymerase at 5 u/µl (Epicentre, Madison, WI) and 2.5 µl ddH<sub>2</sub>O. All components were mixed carefully before 4 µl were aliquoted to 2 µl of each ddNTP termination mix in labeled tubes and covered with mineral oil. The tubes containing this mixture were placed in the thermal controller, and the cycle sequencing reaction was run as described in LI-COR (Lincoln, Neb.) application bulletin 13. Samples were separated on 4.0 % acrylamide gels and analyzed via a LI-COR 4000 LR automated sequencer.

Table 5

20	Name	Sequences (5' to 3')	SEQ ID NO.
25	M13 Forward (-29)/IRD41 Dye-labeled primer	CACGACGTTGTAAAACGAC	16
30	M13 Reverse IRD41 Dye- labeled primer	GGATAACAATTTACACAGG	17

Name	Sequences (5' to 3')	SEQ ID NO.
5' pGEX IRD41 Dye- labeled sequencing primer	GGGCTGGCAAGCCACGTTTGGTG	18
3' pGEX IRD41 Dye- labeled sequencing primer	CCGGGAGCTGCATGTGTCAGAGG	19

#### Colony blotting

Isolates were stab-inoculated into LB agar and incubated overnight at 37° C. Table 3 indicates positive and negative control organisms. Colonies were transferred to charge-modified nylon membranes (QIABRANE Nylon Plus membrane, QIAGEN, Inc., Chatsworth, Calif.) by the method of Grunstein and Hogness (Grunstein *et al.*, Proc. Natl. Acad. Sci. (USA), 72:3961-3065 (1975)). The colonies were lysed and the DNA denatured. Membranes were then stored and sealed in plastic bags (GibcoBRL, Gaithersburg, MD) at 4° C.

#### Total DNA isolation and blotting

Total DNA from all test isolates, including non-*E. coli* strains and control organisms, were isolated by the method of Marmur, J. Mo. Biol., 3:208-218 (1961). Briefly, individual isolates were grown overnight at 37° C with agitation in 50 ml of BHI. Cultures were pelleted by centrifugation (15 minutes at 5000 x g) and the pellets were resuspended in 5 ml of BPES buffer (10 mM HPO<sub>4</sub>, 0.2 M NaCl<sub>2</sub>, and 1 mM EDTA, pH 8.0). Five mg of lysozyme (Sigma Chemical Co., St. Louis, MO)

was added to each suspension and incubated for 45 minutes at 37° C with gentle agitation prior to addition of 0.1 ml of 25 % SDS and 1.0 ml of 5 M sodium perchlorate (Sigma). Suspensions were then incubated for 15 minutes at 65° C with occasional swirling, cooled to room temperature, and 6.5 ml of chloroform isoamyl alcohol (24:1) (Amresco, Solon, OH) was added to each suspension. Tubes were shaken for 5 minutes, and the suspensions pelleted by centrifugation (10 minutes at 600 x g). The aqueous phase was removed to a glass beaker containing two volumes of cold absolute ethanol. Sterile glass rods were used to spool the DNA, which was put into 25 ml plastic, screw-top centrifuge tubes (Fisher Scientific, Chicago, IL). These tubes were centrifuged for 10 minutes at 700 x g, the ethanol decanted, and the DNA allowed to dry. DNA was resuspended in sterile 1 x TE buffer (100 mM Tris-Cl, 10 mM EDTA pH 8.0) (5 Prime → 3 Prime, Inc. Boulder, CO).

Samples of total DNA from each organism were spotted onto nylon, the spots allowed to dry, and the DNA denatured in Denaturation Solution (5 Prime → 3 Prime, Inc., Boulder, CO) for 15 minutes. These "dot" blot membranes were dried briefly on filter paper and were put in Neutralization Solution (5 Prime → 3 Prime, Inc., Boulder, CO) for 15 minutes. Membranes were then placed on filter paper saturated with 2 x SSC (pH 7.0; 5 Prime → 3 Prime, Inc., Boulder, CO) for 5 minutes. All filters were stored in sealed plastic bags (Gibco BRL) at 4° C.

#### Hybridization studies

Membranes were prehybridized in aqueous solution (Prehybridization Solution, 5 Prime → 3 Prime, Inc.) for 4 hours at 68° C and hybridized with the individual, denatured probes at 68° C for 12 hours (Russo *et al.*, Infect. Immun. 61:3578-3582 (1993)). The filters were given two 1 hour washes in 0.1 x SSC (5 Prime → 3 Prime, Inc., Boulder, CO) with 0.1 % SDS (Sigma Chemical Co., St. Louis, MO). Hybridized probes were detected using the protocol in the Genius 1 kit



(Sambrook, Fitch & Maniatis, Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory Press; pp 9.34-9.55 (1989)).

### Capsule staining

- 5 All isolates were examined for the presence of capsule using the method of Hiss, P. H. Jr., J. Exp. Med. 6:317:345 (1905). Briefly, a loopful of normal horse serum was mixed with a loopful of overnight culture on a glass microscopic slide. The film was allowed to air dry and then was heat fixed. The film was covered with crystal violet stain (0.1 g of crystal violet per 100 ml of H<sub>2</sub>O) and warmed until
- 10 steam appeared. The crystal violet was removed with 20% (wt/vol) aqueous copper sulfate solution (CuSO<sub>4</sub>, 5H<sub>2</sub>O; Fisher Scientific), blotted dry, the same covered in oil, a coverslip placed on top of the oil, and examined by light microscopy.

### Biostatistics

- 15 To determine if a factor was characteristic of isolates from sick or healthy birds, the percent positive for each factor in each group was compared using a Z test of proportions (Maxwell, A. E., Analyzing Qualitative Data (Methuen Co., London (1961)). Also, the correlation of *cvaC* with *traT*- and *iss* sequences was calculated for the isolates from sick and healthy birds using the Pearson Product Moment
- 20 Correlation (Maxwell, A. E., Analyzing Qualitative Data, Methuen Co., London (1961)).

### Results

#### Hybridization studies

- 25 Colony blot hybridization procedures were performed on all test and control isolates with each probe. None of the non-*E. coli* strains were detected by any of the prepared probes. Total DNA "dot" blots were found to yield unambiguous results, although the hybridization results were occasionally unclear, for example, false

positives or false negatives obtained with the control organisms. The following results were obtained from either unambiguous colony blots or from "dot" blots.

It was observed that there was no significant difference in the distribution of *traT*, *cvaC*, or *ompA* sequences between the isolates from sick poultry and healthy poultry. All *E. coli* isolates contained *ompA* sequences. There was, however, a highly significant difference in the distribution of *iss* sequences in the isolates of sick poultry and healthy poultry, ( $p < 0.000000001$ ) as the isolates from sick poultry were much more likely to contain *iss* sequences than were the isolates from healthy poultry as shown in Table 6.

Table 6

Gene	% isolates detected with gene		Z	Probability of Z
	Sick Birds	Healthy Birds		
<i>cvaC</i>	66.66	58.9	1.12	0.26
<i>traT</i>	72.7	73.33	0.34	0.73
<i>iss</i>	76.33	23.2	6.69	0.000000001
capsule	1.9	8.9	2.57	0.01

Few encapsulated *E. coli* isolates were detected, and statistical analysis indicated that *E. coli* isolates from healthy poultry were more likely to be encapsulated than were the *E. coli* isolates from sick poultry ( $p < 0.01$ , see Table 6).

Correlations between the occurrence of related sequences within the isolates from healthy or sick poultry were also determined for genes reportedly linked together on ColV plasmids (Waters *et al.*, *Microbiol. Rev.*, 55:437-450 (1991)). For

example, the occurrence of *cvaC*-related sequences for the *E. coli* isolates from healthy and sick poultry was not significantly correlated with the occurrence of *iss*- or *traT*-related sequences.

Approximately 23% of the non-disease-associated avian *E. coli* isolates examined contained *iss*-related sequences. It appears the *iss* isolates of healthy poultry are capable of causing disease, or that the isolates do not express the Iss protein or export the Iss polypeptide to the outer membrane where it is active in protection of the bacterium against host complement.

#### 10 Detection of Iss on the surface of avian *E. coli* isolates

Several methods are available to determine if an *E. coli* isolate expresses Iss. A preferred method indicates the presence of Iss on the outer membrane of the host bacterium where Iss can exert its anti-complement effect. Probing RNA using Northern blotting techniques provides evidence of *iss* transcription, but not translation or presence of Iss in the outer membrane. ELISAs to detect Iss in bacterial lysates are easy to perform and indicate the presence of Iss, but are not useful in determining whether ISS is present on the bacterial outer membrane. Assays useful to determine the location of Iss include fluorescent-antibody techniques or flow cytometry. Preferably, flow cytometry is employed to detect the location of Iss protein due to the accuracy and quantitative nature of the data obtained by this method.

To prepare samples for analysis by flow cytometry, bacteria are grown in Brain Heart Infusion (BHI) broth for 18 hours at 37° C. Bacteria are then washed and resuspended in buffer. Monoclonal antibodies specific for Iss are added to each bacterial suspension and allowed to incubate for 30 minutes. Suspensions are pelleted, washed thoroughly, and incubated with FITC-labeled, goat anti-mouse Ig conjugated antibody for 30 minutes at 0° C. After incubation, suspensions are pelleted, washed thoroughly, fixed with paraformaldehyde, and analyzed by flow cytometry using a FALSCalibur (Becton Dickinson, San Jose, CA) (Otten *et al.*,

Flow Cytometry Analysis Using the Becton Dickinson FACScan, John Wiley & Sons, New York, pp. 5.4.1-5.4.19 (1992)).

### Example 5

#### 5 Construction of an *iss*-based DNA vaccine

Bacterial source of *iss*. The *iss* gene was PCR-amplified from a virulent avian *E. coli* isolate as described in Horne et al., (*Avian Dis.*, 44: 179-184 (2000)).

Cloning of *iss*. The primers for amplification of *iss* were designed using the PrimerSelect program of LaserGene (DNASar, Madison, WI) based on the DNA  
10 sequence in Horne et al. (*Avian Dis.*, 44: 179-184 (2000); GenBank Acc. No. AF042279; SEQ ID NO:1). To facilitate cloning, these primers were modified to add restriction sites (*Bam*HI on the upper primer and *Eco*RI on the lower primer at the 5' ends). The modified nucleotides are present in the primers as non-italicized text, and the italicized nucleotides hybridize with nucleotides present in SEQ ID  
15 NO:1 or the complement thereto. The primers were *iss* pVAX1 Upper, 5'AGTGGGGATCCTAACAATGCAGGATAATAAGATGA (SEQ ID NO:23); and *iss* pVAX1 Lower, 5'ATGCGGAATTCTGTAGGGAGCCCAGAAGTA (SEQ ID NO:24).

Based on the unmodified primers, amplification conditions recommended by  
20 PrimerSelect were determined, and these were used to amplify *iss* from the virulent avian *E. coli*. DNA template from the *E. coli* isolate used in the amplification was prepared as follows. A colony was lysed in a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 50 µg/ml proteinase K by incubation at 55°C for 10 minutes followed by incubation at 80°C for 10 minutes. After dilution with a 2x  
25 volume of ddH<sub>2</sub>O, cellular debris were removed by a brief centrifugation (30 seconds at 10,000 x g), and the supernatant containing the DNA was transferred to a fresh tube. Five microliters of the DNA sample was added to a 45 µl reaction volume of Master Mix in a 200 µl thin walled microfuge tube. Master Mix was 27 µl of ddH<sub>2</sub>O, 5 µl of 0.1 mM of the primer *iss* pVAX1 Upper, 0.5 µl of 0.1 mM of

the primer *iss* pVAX1 Lower, 0.25 Amplitaq (5 Units/ml)(Ampligene Kit, Perkin-Elver, Foster City, CA), and 4 µl of 25 mM MgCl<sub>2</sub>. A Perkin-Elmer PE2400 thermocycler was used to amplify *iss*. The amplification protocol used was 95°C for 5 minutes; 9 cycles of 95°C for 1 minute, 51.6°C for 30 seconds, and 72°C for 30 seconds; 25 cycles of 94°C for 30 seconds, 51.6°C for 30 seconds, and 72°C for 30 seconds; 72°C for 7 minutes; 4°C soak.

Using this procedure, a 400 base pair amplicon was generated. This amplicon was gel purified and removed from the low melt agarose using Wizard PCR Clean Up Kit (Promega, Madiosn, WI). The *iss* amplicon was digested with EcoRI and BamHI to prepare for cloning. At the same time, pVAX1, a plasmid specifically designed for use in DNA vaccines (Invitrogen, Carlsbad, CA) was also prepared for cloning. The digests were purified with Wizard DNA Clean UP Kits (Promega) according to the manufacturer's recommendations. *iss* was ligated into pVAX1 in 1:1 and 3:1 ratios of molar ends (Sambrook et al. Molecular Cloning, Second Edition, Cold Spring Harbor Laboratory, Plainview, New York (1989)) using T4 DNA ligase (GIBCO BRL, Gaithersburg, MD). Ligations were transformed into chemically competent bacterial cells (XL-1 Blue, Stratagene, La Jolla, CA). Transformants were selected on kanamycin (50 µg/ml kanamycin, Amresco, Solon, OH). Kanamycin-resistant colonies were transferred to LB broth containing 50 µg/ml kanamycin. After overnight growth, some culture was reserved, and some was subjected to DNA isolation using Wizard MiniPreps (Promega). DNA was restricted with BamHI and XbaI (New England Biolabs, Beverly, MA). Desired clones were presumptively identified by the size of DNA fragments generated following plasmid restriction. The identity of the insert was confirmed by sequence analysis. The insert region was sequenced using IRD800 labeled T7 promoter/primer (TAATACGACTCACTATAGGG, SEQ ID NO:25) and BGH reverse primer (TAGAAGGCACAGTCGAGG, SEQ ID NO:26) (LICOR, Lincoln, NE) with the Sequitherm EXCEL II (Epicentre, Madison, WI) cycle

sequencing kit on a LI-COR 4000LS automated DNA sequencer. The *iss* vaccine construct can be seen in Figure 6.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (e.g., GenBank amino acid and nucleotide  
5 sequence submissions) cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.